8-20-2020 3:00 PM

# Elucidating the Role of Host Tim-1 in Anti-tumour Immunity

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

# ABSTRACT

T cell immunoglobulin and mucin domain-1 (Tim-1) is expressed by CD4+ T cells, invariant natural killer T (iNKT) cells, and regulatory B cells (Bregs). Tim-1 expression on CD4+ T cells and iNKT cells typically promote proinflammatory responses, while Tim-1-expressing Bregs are immunosuppressive. However, the potential role of Tim-1 in anti-tumour immunity is unclear. To investigate the effects of Tim-1 on B16F1 melanoma and MC38 colorectal adenocarcinoma models, I compared tumour growth and survival between Tim-1-expressing (wild-type) and Tim-1-deficient mice. Using B16F1 melanoma, I demonstrated that Tim-1-deficient mice exhibited delayed tumour growth and improved survival, compared to wild-type mice. When immunized with heat-killed B16F1 melanoma cells, decreased frequencies of Bregs were found in Tim-1-deficient mice. These data suggest that Tim-1 expression may promote tumour growth by inducing Bregs. Therefore, anti-cancer treatments aimed at suppressing the function of Tim-1-expressing Bregs may be of therapeutic value.

# LAY SUMMARY

Cancer is a disorder that describes abnormal cell growth or tumours that can interfere with the growth and development of normal cells. The high prevalence and mortality rate of cancer makes it a serious global burden. Throughout the years, various therapies have been developed to treat cancer, but curative therapies remain elusive for most cancers, including colon cancer and melanoma (skin cancer).

The immune system plays a key role in detecting and eliminating cancer throughout life. Developing immunity against cancer (anti-cancer immunity), much like immunity against viruses, has the potential to be curative. While immune cells utilize a variety of mechanisms to kill cancer cells, the existence of cancer cells indicates that cancer cells learn to evade the host's immune system. Cancer cells can escape the immune system by preventing detection or infiltration of immune cells, or by inhibiting the function of immune cells that have infiltrated the tumour. In addition, cancer cells can recruit immune cells that modulate and suppresses the immune system. Therefore, further understanding the workings of anti-cancer immunity may lead to potentially curative cancer therapies.

This study focuses on T cell immunoglobulin and mucin domain-1 (Tim-1). Tim-1 is a protein found in some important anti-cancer immune cells. However, the role of Tim-1 in anti-cancer immune responses is unclear.

In this study, I tested the effect of host Tim-1 on cancer growth and survival by implanting mouse colon or melanoma tumours into normal mice or mice lacking the gene for Tim-1. I found that mice lacking the Tim-1 gene have improved survival and delayed tumour growth, compared to mice expressing the Tim-1 gene. As well, examination of anti-cancer

immune responses in the mice lacking Tim-1 revealed that they have fewer B cells, a type of immune cell. I conclude that host Tim-1 impairs anti-cancer immune responses. Furthermore, the inhibition of host Tim-1 on B cells may be a new strategy for improving or augmenting host anti-cancer immune response in patients.

# KEYWORDS

T cell Immunoglobulin and Mucin Domain-1, Tim-1, Tumour Immunology, Regulatory B cells, Bregs, Melanoma

# CO-AUTHORSHIP STATEMENT

Brad Shrum (research technician) from Gunaratnam laboratory at the Department of Microbiology and Immunology at the University of Western Ontario assisted in the intraperitoneal injections of Tim-1-expressing and Tim-1-deficient C57BL/6 mice.

Dr. Xizhong Zhang (research associate) from Gunaratnam laboratory at the Department of Microbiology and Immunology at the University of Western Ontario performed the PCR genotyping of Tim-1-expressing and Tim-1-deficient C57BL/6 mice.

Weihua (Winnie) Liu from the Department of Pathology and Laboratory Medicine at the University of Western Ontario processed the tumour samples, prepared slides, and stained them for histopathological analysis.

Dr. Manal Gabril (pathologist) from the Department of Pathology and Laboratory Medicine at the University of Western Ontario analyzed tumour samples for necrosis.

# ACKNOWLEDGEMENTS

First, I would like to thank my advisory committee members, Dr. Greg Dekaban and Dr. Zhuxu Zhang, for their constant guidance throughout my graduate studies. Their advice and feedback proved to be invaluable to me in during my graduate research work and helped immensely in improving my experimental planning and preparation of this thesis.

Thank you to all past and present lab members, who provided the support and assistance needed for this project. To my lab manager, Brad Shrum, thank you for always entertaining my countless questions (lab-related or not), handling my mice when they were particularly unfriendly, and overall, for being a great mentor. Thank you to Dr. Xizhong Zhang and Dr. Elena Tutunea-Fatan for always being there to help me troubleshoot my experiments and offering me insightful advice. Thank you to Ji Yun Lee and Demitra Yotis for helping me throughout my graduate studies and keeping things running smoothly.

To the members of the Matthew Mailing Centre for Translational Transplant Studies, Dr. Jacqueline Arp and Patrick McLeod, thank you for helping me with the IncuCyte system and providing your expertise.

Finally, I would like to thank my graduate supervisor, Dr. Lakshman Gunaratnam, for not only overseeing my project, but for providing round-the-clock mentorship. Dr. Gunaratnam has consistently encouraged me to think outside the box, persevere through hardships, and work hard to accomplish my goals. I am extremely grateful to have joined the Dr. Gunaratnam lab and I will continue to apply everything I have learned under his guidance in my future endeavours.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **APC** | antigen presenting cell |
| **Breg** | regulatory B cell |
| **DMEM** | Dulbecco's modified Eagle medium |
| **FBS** | fetal bovine serum |
| **H&E** | Hematoxylin & Eosin |
| **HBSS** | Hanks' balanced salt solution |
| **IFN** | interferon |
| **Ig** | immunoglobulin |
| **IL** | interleukin |
| **iNKT** | invariant natural killer T cell |
| **MDSC** | myeloid-derived suppressor cell |
| **MHC** | major histocompatibility complex |
| **NK** | natural killer |
| **PBS** | phosphate buffered solution |
| **PS** | phosphatidylserine |
| **PTEC** | proximal tubular epithelial cell |
| **RPMI** | Roswell Park Memorial Institute medium |

| | |
|---|---|
| **TGF** | transforming growth factor |
| **Th** | T helper |
| **Tim-1** | T cell immunoglobulin and mucin domain-1 |
| **TNF** | tumour necrosis factor |
| **TRAIL** | TNF-related apoptosis-inducing ligand |
| **Treg** | regulatory T cell |
| **UWO** | University of Western Ontario |

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# Chapter 1

## 1 INTRODUCTION

### 1.1 Cancer

Cancer is described as a disorder that results from genetic mutations or epigenetic alterations leading to aberrant cell growth. The high prevalence and mortality rate of cancer has made it an enormous global burden throughout the years. Cancer is the second leading cause of death in the world, with approximately one in six deaths being attributed to cancer [1, 2]. While innumerable efforts have been made to improve cancer therapies, the severity of this disease remains, and an effective solution remains elusive.

### 1.2 The Immune System and Cancer

The immune system is essential in recognizing and eradicating tumours. Tumour cells can lack 'self' molecules, such as MHC class I, or express neoantigens (new antigens formed as a result of tumour mutations that are not recognized by the immune system), and the immune system detects these abnormal cells as 'non-self' [3]. Subsequently, the immune system employs various cell types and mechanisms to eliminate tumour cells. Some key players in anti-tumour immunity include T cells, Natural Killer (NK) cells, invariant natural killer T (iNKT) cells, and B cells [4-8].

#### 1.2.1 CD8+ T cells

T cells are a vital component of the adaptive immune system, and T cells can develop into CD8+ T cells or CD4+ T cells. CD8+ T cells are cytotoxic lymphocytes restricted to recognizing antigens presented on major histocompatibility complex (MHC) class I molecules expressed by antigen presenting cells (APCs). Once activated by antigen-

specific recognition, CD8+ T cells clonally expand into cytotoxic effector cells. Cytotoxic effects of CD8+ T cells are predominantly mediated by the release of perforin and granzyme, which are cytotoxins that trigger apoptosis in target cells, including susceptible tumour cells [5, 9]. Perforin functions to create pores in the cytoplasmic membrane of the target cell, allowing granzyme to enter the cell and activate apoptosis.

In addition to its cytotoxic effects, CD8+ T cells enhance anti-tumour immune responses through cytokine functions. CD8+ T cells produce the proteins tumour necrosis factor (TNF)-α and TNF-related apoptosis inducing ligand (TRAIL), which induces apoptosis in tumour cells [5, 6]. TNF is a highly pleiotropic cytokine and plays various roles in tumour immunosurveillance. The effects of TNF include apoptosis, necrosis, immune cell activation, cell migration, and even angiogenesis. As a result, TNF may promote tumour killing or tumour progression [10]. CD8+ T cells also produce IFN-γ to promote the activation and recruitment of NK cells, macrophages, and neutrophils [11]. IFN-γ can enhance the expression of MHC class I antigens by tumour cells, which improves detection and killing by CD8+ T cells [12]. Finally, IFN-γ can potentially act directly on tumour cells to inhibit growth [11]. To highlight the importance of IFN-γ in anti-tumour immune responses, a landmark study by Kaplan, et al. demonstrated that IFN-γ receptor-deficient mice are prone to accelerated tumour growth when compared to wild-type mice, in both chemically-induced and spontaneous tumour models [13]. These data serve to emphasize the critical role of CD8+ T cells in anti-tumour immunity.

### 1.2.2 CD4+ T cells

While CD8+ T cells recognize antigens presented on MHC class I molecules and directly target tumours, CD4+ T cells are restricted to recognizing antigens presented on MHC

class II molecules by APCs. CD4+ T cells further develop into specific T helper (Th) subsets, including Th1, Th2, Th17, and regulatory T cells (Tregs). Th1 cells typically promote immune responses against intracellular pathogens, while Th2 cells generate immune responses against extracellular pathogens and may be implicated in promoting allergic responses. Studies have suggested that Th1 cells can play a role in anti-tumour immune responses by priming CD8+ T cells, recruiting NK cells, and inhibiting angiogenesis [7]. On the other hand, Th2 cells may function to promote or inhibit tumour growth [14]. The production of IL-4 by Th2 cells has been observed to recruit immune cells to the tumour, and the adoptive transfer of Th2 cells promotes the elimination of cytotoxic T lymphocyte resistant-melanoma in mice [7, 14]. In contrast, IL-5-producing Th2 cells were associated with progressive tumour growth in renal cell carcinoma and melanoma in humans [7, 15]. Similarly, Th17 cells, which promote immune responses against extracellular bacteria and fungi, can play a contradicting role in anti-tumour immune responses. In some tumour studies, the cytokine production by Th17 cells was correlated with tumour progression and angiogenesis [7, 16], while other studies have shown that the transfer of Th17 cells is associated with enhanced CD8+ T cell activation and immune cell recruitment to the tumour [7, 17, 18]. Collectively, Th1, Th2, and Th17 cells are generally proinflammatory cells that elicit immune responses potentially favouring anti-tumour immunity.

Interestingly, some studies have shown that CD4+ T cells can even develop direct cytotoxic activity against tumour cells [9, 19, 20]. In a study of melanoma, the transfer of naïve, tumour-reactive CD4+ T cells into lymphocyte-depleted mice resulted in tumour rejection, which was further enhanced by CTLA-4 blockade. This tumour rejection was found to be

the result of acquired cytotoxic CD4+ T cell activity, directly targeting tumour cells presenting antigens on MHC class II molecules. These cytotoxic CD4+ T cells were proposed to function like CD8+ T cells, using granzyme-containing granules to cause tumour cell death [19].

In contrast, regulatory T cells (Tregs) are a subset of CD4+ T cells that tend to be associated with anti-inflammatory and immunosuppressive responses [21]. Tregs, responding to chemokines, can be recruited to the tumour and suppress immune cells by producing immunosuppressive cytokines and metabolites, inhibiting APC function, using checkpoint molecules to impair cytotoxic T cell function, and killing effector T cells [21]. Cumulatively, these effects drastically hinder the elimination of tumours, and studies have established that high Treg:CD8 ratios in tumours are associated with poorer patient survival [7]. Therefore, the role of CD4+ T cells in anti-tumour immunity can be variable and may depend on the CD4 subtype and the context of the tumour microenvironment.

### 1.2.3 NK cells

NK cells are a component of the innate immune system and are lymphoid cells that lack surface T cell receptors or antigen-specific receptors [22]. They are known to kill virally infected cells and tumour cells via NK cell-mediated cytotoxicity and cytokine production. Sharing similar functions with CD8+ T cells, NK cells utilize perforin and granzyme to kill infected cells, as well as employing TRAIL to target tumour cells [5]. In addition, NK cells produce IFN- γ, which promotes the function of some innate cells and may directly inhibit tumour growth [11], as previously mentioned. In contrast, specific to NK cell function, NK cells can recognize and kill abnormal cells without the need for prior activation [23]. The ‘missing self’ hypothesis states that healthy cells will express self-MHC class I molecules,

while abnormal cells, including some tumour cells, lack the expression of self-MHC molecules. These abnormal cells are subsequently detected and eradicated by NK cells [24]. However, some tumour cells expressing self-MHC molecules can still be cleared by NK cells, while erythrocytes, lacking in self-MHC-expression, can avoid NK cell killing. This suggests that the activation or inhibition of NK cell activity is not dependent solely on self-MHC expression or a single induction signal. Rather, NK cell activity relies on, and is dictated by, a sum of activation and inhibition signals mediated by different cell surface receptors [23]. Of note, the infiltration of NK cells in solid tumours is associated with an improved prognosis in patients [25]. Thus, NK cells are a crucial component in tumour immunosurveillance.

### 1.2.4 iNKT cells

iNKT cells are a subset of T cells that share characteristics of both NK cells and T cells, thus they are considered to bridge innate and adaptive immunity [8]. Along with various NK cell surface molecules, iNKT cells express invariant T cell receptors that recognize a diverse range of lipid antigens in a CD1d-restricted manner. Recognition of lipid antigens by iNKT cells results in the production of immunostimulatory cytokines, which enhances the ability of other immune cells, such as APCs, B cells, and T cells, to detect and eradicate tumours. iNKT cells can be activated directly by some CD1d-expressing tumour cells presenting lipid antigens. Alternatively, activation of iNKT cells occurs via presentation of tumour lipids by APCs [26, 27]. More importantly, human iNKT cells have been shown to have cytotoxic effects against CD1d-expressing osteosarcoma tumours [26, 28]. Osteosarcoma cells expressing CD1d were effectively recognized and killed by iNKT cells, whereas osteosarcoma cells without CD1d expression were not eliminated [26, 28]. In

addition, a model of murine breast cancer showed that the downregulation of CD1d expression by tumour cells inhibited iNKT cell-mediated killing and promoted metastases [26, 29]. These data demonstrate the importance of iNKT cells in anti-tumour immunity.

### 1.2.5 B cells

Finally, the role of B cells in cancer has been widely disputed due to their dual effects on promoting and inhibiting anti-tumour immune responses [4]. Antibodies produced by plasma cells, which are derived from B cells, can recognize tumour antigens to induce complement-mediated tumour killing, Fc-mediated phagocytosis, and antibody-dependent cell-mediated cytotoxicity [4]. These mechanisms promote the recognition of tumours by phagocytes and NK cells. Additionally, B cells function as APCs, serving to activate T cells and potentially bolstering anti-cancer responses. Of clinical relevance, the presence of B cells in tumour infiltrates have been correlated with improved patient survival in some tumours [4]. However, B cells have also been shown to suppress immune responses. Antibodies bound to antigens can form immune complexes, which induce the production of myeloid-derived suppressor cells (MDSCs), leading to inhibition of CD4+ and CD8+ T cell activity [4, 30-32]. A subset of B cells, regulatory B cells (Bregs), are immunomodulatory cells critical for regulating immune homeostasis. Bregs produce a variety of cytokines, including IL-10 and TGF-$\beta$ to inhibit proinflammatory cells and promote the generation of Tregs, MDSCs, and other immunosuppressive cells [4, 30-32]. It has been shown that using IL-10, Bregs suppressed inflammation in models of colitis, arthritis, and experimental autoimmune encephalomyelitis [33-36]. More importantly, it was reported that IL-10 production by Bregs can suppress the phagocytic capacity of macrophages and promote tumour progression [31, 37, 38]. Additionally, in a murine

model of breast cancer, anti-CD20 was used to deplete B cells. Since Bregs express low levels of CD20, Bregs were not depleted and tumour metastasis was paradoxically enhanced in this study [31, 37, 39]. Thus, while B cells can promote tumour killing by serving as APCs to activate tumour-specific T cells, some subsets (e.g. Bregs) can be detrimental to anti-tumour responses.

## 1.3 Immunoediting and Immune Evasion

While the immune system may seem efficient at detecting and eradicating tumours (immunosurveillance), one of the fundamental hallmarks of cancer is the ability to evade the immune system [2]. It can be argued that a tumour cannot exist without the ability to evade the host immune response. This involves the concept of immunoediting, which describes immunosurveillance and cancer progression via three phases – elimination, equilibrium, and escape [40, 41]. The elimination phase requires immune cells to successfully detect and eradicate tumour cells completely. Equilibrium describes a phase of dormancy, where there is a balance between tumour elimination and tumour progression. While the immune system remains active to kill tumour cells, tumour elimination is not complete. In the equilibrium phase, the immune system essentially selects for tumour cells with the ability to avoid immune detection and elimination. However, tumour cells can be maintained in the equilibrium phase for long periods of time. Some tumour cells can no longer be controlled or maintained by the immune system and enters the escape phase, where tumours are clinically detectable [40, 41].

In order to escape the immune system, tumour cells frequently inhibit the host lymphocytes that directly mediate or regulate anti-tumour immunity, including T cells, NK cells, and iNKT cells [42]. For instance, tumours create an immunosuppressive microenvironment to

evade the immune system by recruiting regulatory T cells, regulatory B cells, and myeloid-derived suppressor cells, stimulating the production of immunosuppressive cytokines (eg. IL-10 and TGF-$\beta$), and/or expressing immune checkpoint molecules to inhibit activated lymphocytes [43-45]. Immune checkpoint molecules are normally expressed by immune cells to suppress and regulate immune responses. However, some tumour cells exploit this mechanism and use immune checkpoint molecules to evade the immune system [46]. In the past few years, dramatic improvements in anti-cancer therapies have arisen due to the discovery of checkpoint inhibitors (eg. Nivolumab) that serve to block these immune evasion pathways. However, despite their effectiveness, only a small fraction of patients respond to them [47]. Therefore, discovering novel mechanisms of anti-cancer immunity will be crucial to developing future cancer therapies.

## 1.4 T cell immunoglobulin and mucin domain-1 (Tim-1)

Tim-1 is a type I transmembrane glycoprotein [48, 49] that is expressed in a variety of cell types in the host, including both immune cells and parenchymal cells. Tim-1 functions as a receptor for ligands such as Tim-4 and phosphatidylserine (PS), with Tim-4 being predominantly expressed on APCs and PS expressed on early apoptotic cells [50, 51]. In addition, Tim-1 functions as a receptor for Hepatitis A virus, hence, its gene is also referred to as Hepatitis A Virus Receptor 1 (*HAVCR1)* [52, 53].

In the kidney, Tim-1 is referred to as kidney injury molecule-1 (Kim-1) and is highly upregulated on proximal tubular epithelial cells (PTECs) during acute kidney injury [48]. Tim-1 is a receptor for phosphatidylserine (PS), which functions as an “eat me” signal on apoptotic and necrotic cells, and Tim-1 ligation induces PTECs to phagocytose apoptotic and necrotic cells [49, 54, 55]. This process aids in the clearance of dying cells and reduces

inflammation [56], which has been shown to promote tissue repair. Mice transplanted with syngeneic Tim-1-expressing donor kidneys exhibited improved graft outcome and greater survival compared to mice receiving Tim-1-deficient donor kidneys, demonstrating the importance of Tim-1 expression in renal repair during transplantation [57, 58].

## 1.5 Tim-1 in the Immune System

In the immune system, lymphocytes expressing Tim-1 include CD4+ T cells, iNKT cells, and Bregs [59-62]. The TIM gene family was originally discovered in a mouse model of asthma, where Tim-1 expression on T cells was shown to influence the regulation of atopic diseases such as asthma. Some TIM-1 polymorphisms in humans were also associated with an increased risk of asthma [63, 64]. Protection against atopy in humans was associated with prior infection with Hepatitis A virus, the receptor for which is encoded by the Tim-1 gene, *HAVCR1* [52, 65]. This finding was essential in supporting the hygiene hypothesis, which states that the increase of allergic and autoimmune diseases is correlated to the decrease of infectious diseases [66, 67].

Tim-1 expression is upregulated on CD4+ T cells following T cell receptor (TCR) engagement by cognate peptide-MHC interactions on APCs and functions as a costimulatory molecule [52, 68]. Interaction between Tim-1 on activated CD4+ T cells and Tim-4 on APCs can promote T cell activation, proliferation, and cytokine production [50, 69, 70]. Tim-1 is preferentially expressed by Th2 cells, compared to Th1 and Th17 cells [59, 69, 71-73]. Moreover, Tim-1 has been implicated in promoting the trafficking of Th1 and Th17 cells [74]. P-selectin, an adhesion molecule expressed on the surface of activated endothelial cells, is a potential ligand for Tim-1 and can mediate the rolling and tethering of lymphocytes via Tim-1. Th1 and Th17 cells expressing Tim-1 with a deficient mucin

domain exhibited reduced tethering and adherence to P-selectin. In a contact hypersensitivity model, mice with Th1 cells that have mucin domain-deficient Tim-1 were observed to experience a decreased recruitment of Th1 cells to inflamed skin [74]. In addition, mice with Th1 cells that express mucin domain-deficient Tim-1 exhibited decreased severity of experimental autoimmune encephalomyelitis [74].Therefore, the expression of Tim-1 could theoretically enhance anti-tumour immunity by inducing the activation of CD4+ T cells, which can subsequently promote inflammatory responses, improve lymphocyte trafficking, and lead to increased CD8+ T cell activation.

Tim-1 is constitutively expressed on iNKT cells and binds PS present on apoptotic cells [75]. This interaction not only leads to iNKT cell activation, proliferation, and cytokine production, but the ligation of PS to Tim-1 on pulmonary iNKT cells can induce airway hyperreactivity, which is a critical feature of asthma [75]. More importantly, iNKT cells have been implicated in promoting an anti-tumour immune response [26]. Studies have shown that mice deficient in iNKT cells exhibit greater tumour growth [76, 77], and in a variety of solid malignancies in humans, diminished iNKT cell function and cell number was observed [78-80]. Notably, iNKT cells can directly kill tumour cells by secreting perforin and granzyme [81], and activation of iNKT cells promotes the induction of CD4+ T cell, CD8+ T cell, and NK cell anti-tumour responses [82-84].

Tim-1 expression is upregulated on B cells upon B cell receptor signalling, and Tim-1 subsequently interacts with PS on apoptotic cells [85]. This leads to B cells differentiating into IL-10-producing Bregs, which are essential for regulating and suppressing immune responses [85, 86]. It has been postulated that Tim-1 is predominantly expressed by B cells, rather than T cells, with approximately 70% of IL-10-producing Bregs expressing Tim-1

[87, 88]. Tim-1 expression on B cells can be induced using anti-IgM *in vitro*, which suggests that Tim-1 expression on B cells is dependent on B cell receptor signalling [62]. It has been shown that mice lacking Tim-1 have a dramatic reduction in the number of IL-10-producing Bregs, which leads to T cell hyper-responsiveness and susceptibility to rejection in cardiac transplantation [85]. In addition, separate studies have demonstrated that the adoptive transfer of Tim-1-expressing Bregs prolonged allograft survival of islet transplants [87] and reduced the severity of experimental autoimmune encephalomyelitis [86]. More importantly, Ding et al. demonstrated accelerated tumour growth in B cell-deficient mice inoculated subcutaneously with B16F10 melanoma and receiving a transfer of syngeneic Tim-1-expressing B cells from B16F10 immunized mice [89]. These findings further suggest that the role of Tim-1 expression on Bregs is immunosuppressive and may potentially inhibit anti-tumour immune responses, but this has not yet been well studied.

Although the versatile role of Tim-1 has been established in parenchymal and immune cells, the potential role of Tim-1 on anti-tumour immunity is not yet known.

## 1.6 RATIONALE AND HYPOTHESIS

In this study, the murine tumour models, B16 melanoma and MC38 colorectal adenocarcinoma were used to study anti-cancer immune responses. They are widely used in tumour studies, grow well as a subcutaneous tumour, and are variably immunogenic (able to induce an immune response) [90-92]. Thus, these tumours can be readily monitored, and immune responses can be analyzed.

B16 melanoma is a spontaneously derived C57BL/6 cell line, commonly used to study malignant melanoma, its metastatic properties, and various treatment options. Several

variants of B16 melanoma are commercially available, including B16F1 (parental), B16F0 (non-metastatic), and B16F10 (highly metastatic) [90]. In this study, the parental line, B16F1, will be used to investigate anti-tumour immunity in the absence of metastases.

To further support my findings, tumour growth and survival using MC38 colorectal adenocarcinoma were studied. MC38 colorectal adenocarcinoma was derived from a chemically induced adenocarcinoma in a C57BL/6 mouse, and has been shown to be moderately immunogenic [93]. These murine tumour models were implemented to test the robustness of my hypothesis and to test the ability to generalize my findings to other cancers.

Since the expression of Tim-1 affects the activation and/or function of CD4+ T cells, iNKT cells, and Bregs in various capacities, I hypothesized that the proinflammatory responses of CD4+ T cells and iNKT cells induced by Tim-1 would exceed the immunosuppressive effects of Tim-1-expressing Bregs. I expected to observe decreased tumour growth and improved survival in Tim-1-expressing mice inoculated with subcutaneous tumours, compared to Tim-1-deficient mice.

# Chapter 2

## 2 MATERIALS AND METHODS

### 2.1 Mice

Male, 8-10 week old, wild-type (Tim-1-expressing) C57BL/6 (B6; H-$2^b$) mice were purchased from the Charles River Laboratory. Tim-1-deficient C57BL/6 mice were generated and generously provided by Dr. Andrew N.J. McKenzie (MRC laboratory of Molecular Biology, Cambridge, UK). Tim-1-deficient mice were generated by targeted disruption of the mouse Tim-1 (*HAVCR1*$^{-/-}$) gene in mouse embryonic stem cells, with no change in the overall phenotype compared to wild-type C57BL/6 mice [62]. The Tim-1-deficient genotype was confirmed by Dr. Xizhong Zhang (Gunaratnam lab) using PCR genotyping. Mice were maintained in the Animal Care and Veterinary Services facility at the University of Western Ontario (UWO) and food and water were provided ad libitum. All animal protocols were approved by the Animal Care Committee at UWO (Protocol number: 2018-147) (Appendix A).

### 2.2 Murine Tumour Cell Culture

B16F1 melanoma cells were purchased from the American Type Culture Collection (Manassas, Virginia, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (Thermo Fisher Scientific).

MC38 colorectal adenocarcinoma cells were generously provided by Dr. James Koropatnick (The University of Western Ontario). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% P/S.

Unless otherwise stated, all cell lines were cultured at 37ºC and 5% $CO_2$. All cell lines were washed with 1x PBS and passaged for a maximum of 10 times using 0.25% trypsin (Life Technologies).

## 2.3 Subcutaneous Tumour Models

8-10 week old, male, Tim-1-expressing and Tim-1-deficient C57BL/6 mice were anesthetized with isoflurane and subcutaneously injected in the right flanks with B16F1 melanoma ($5x10^5$ cells), MC38 colorectal adenocarcinoma ($1x10^5$ cells), or B16F10-OVA melanoma ($5x10^5$ cells) in 100ul of Hanks' Balanced Salt Solution (HBSS). Tumours were measured with calipers and tumour volume was calculated with the formula: $mm^3$=(length x $width^2$)/2. Endpoints were determined by incidence of cachexia, tumour ulceration, or tumour diameter exceeding 10mm, as per standard operating procedures of the Animal Care and Veterinary Services at UWO.

## 2.4 Histology

Excised tumours were washed with 1X PBS and fixed in 10% neutral buffered formalin for 48 hours. Fixed samples were sent to a laboratory technician (Weihua Liu from the Department of Pathology and Laboratory Medicine at UWO) for paraffin embedding and staining with Hematoxylin & Eosin (H&E). Stained tumour sections were analyzed by a pathologist (Dr. Manal Gabril, MD, MSc, FRCPC) in a blinded fashion and percent necrosis was scored using Suzuki's histological criteria [94]. Suzuki's criteria scores necrosis as a percentage, ranging from none (0%), minimal (single-cell necrosis), mild (< 30%), moderate (30-60%), to severe (> 60%).

## 2.5 Isolation of Splenocytes

Excised spleens were washed with 1X PBS and mechanically homogenized through a 40um cell strainer (Fisher Scientific) in 1X PBS. The cell suspensions were centrifuged (Thermo Scientific Medifuge Benchtop Centrifuge) at 500rcf for 5 minutes at 4°C and resuspended with 1ml of ACK lysis buffer for each spleen for 1 minute. Splenocytes were washed twice with 1X PBS. For flow cytometry, splenocytes were cryopreserved in 90% FBS and 10% DMSO at -80°C, and when needed, cryopreserved splenocytes were rapidly thawed in RPMI media (with 10% FBS) at 37°C.

## 2.6 Immunization with Heat-Killed B16F1 Melanoma

B16F1 melanoma cells ($5x10^6$ cells/ml) suspended in 1X PBS were heat-killed in a water bath at 65°C for 20 minutes. Heat-killed cells were kept on ice until injections were performed. Mice were anesthetized using isoflurane and $5x10^5$ heat-killed B16F1 melanoma cells were subcutaneously injected into the right flanks of Tim-1-expressing and Tim-1-deficient mice. Mice were euthanized on day 7 post-immunization and splenocytes were isolated and process as described above.

## 2.7 Flow Cytometry

All staining procedures were performed in 1X PBS with 2% FBS at 4°C. To block non-specific binding, single-cell suspensions were incubated with Purified Rat Anti-Mouse CD16/CD32 (BD Biosciences) for 10 minutes. Without washing, cell surface markers were stained by incubating with fluorochrome-conjugated antibodies for 30 minutes and resuspended in 1xPBS with 2% FBS. Intracellular staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Fluorochrome-conjugated

antibodies used in this study can be found in Table 1. Negative controls were established using isotype-matched controls. Cells were analyzed on a CytoFLEX S flow cytometer (Beckman Coulter), and data obtained were analyzed using FlowJo software (BD Life Sciences).

*Table 1. Antibodies used for flow cytometry*

| Antibody | Fluorochrome | Clone | Source |
|---|---|---|---|
| **Anti-mouse TCR-β** | BV605 | H57-597 | BioLegend |
| **Anti-mouse CD8a** | PE-Cy7 | 53-6.7 | eBioscience |
| **Anti-mouse CD4** | BV421 | GK1.5 | BioLegend |
| **Anti-mouse CD279 (PD-1)** | APC | RMP1-30 | eBioscience |
| **Anti-mouse T-bet** | APC | 4B10 | BioLegend |
| **Anti-mouse GATA3** | APC | 16E10A23 | BioLegend |
| **Anti-mouse FOXP3** | APC | FJK-16s | eBioscience |
| **Anti-mouse NK1.1** | APC | PK136 | eBioscience |
| **Anti-mouse CD19** | APC | 6D5 | BioLegend |
| **Anti-mouse CD1d** | PE-Cy7 | 1B1 | BioLegend |
| **Anti-mouse CD5** | BV421 | 53-7.3 | BioLegend |
| **Anti-mouse CD11b** | FITC | M1/70 | BioLegend |
| **Anti-mouse Ly-6C** | BV605 | HK1.4 | BioLegend |
| **Anti-mouse Ly-6G** | BV421 | 1A8 | BioLegend |

## 2.8 IncuCyte Immune Cell Killing Assay

On day 0, B16F1 melanoma cells were seeded (1000, 2000, or 3000 cells in 100ul of RPMI media + 10% FBS + 1% P/S per well) into a 96-well flat bottom plate (Corning 3595) to achieve a cell confluency of ~20% on day 1. On day 1, media was gently removed from each well. To each well, 100nM of Sytox Green Nucleic Acid Stain (Thermofisher) and effector cells (splenocytes from Tim-1-expressing or Tim-1-deficient mice) in DMEM were added to achieve a total assay volume of 200ul. Two target-to-effector cell ratios were used (1:5 and 1:10). Negative controls received no effector cells (media only) and positive controls received 1% Triton X-100 (Sigma Aldrich). The assay plate was placed into the IncuCyte Live-Cell Analysis System (Essen Bioscience) to be incubated at 37ºC and 5% $CO_2$ and fluorescent images of each well were taken hourly at 4x magnification for 24 hours.

## 2.9 *In vivo* RMT1-10 Treatment

Anti-mouse Tim-1 (RMT1-10; Bio X Cell) or control rat IgG2a (Bio X Cell) was administered intraperitoneally at 300ug in *InVivo*Pure pH 7.0 Dilution Buffer (Bio X Cell) on days −1, 0, and every 3 days relative to the day of subcutaneous tumour injections.

## 2.10 Statistical Analyses

Statistical analyses used a log-rank (Mantel Cox) test, a repeated measures ANOVA, a Mann-Whitney test, or an unpaired two-tailed Student's t-test, as indicated. Differences were considered significant at p values $<0.05$.

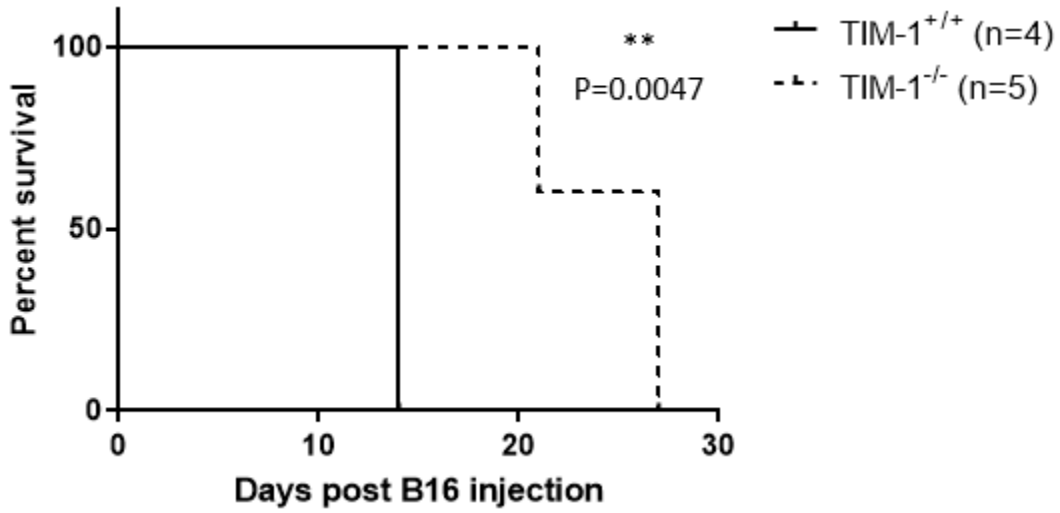
# Chapter 3

## 3 RESULTS

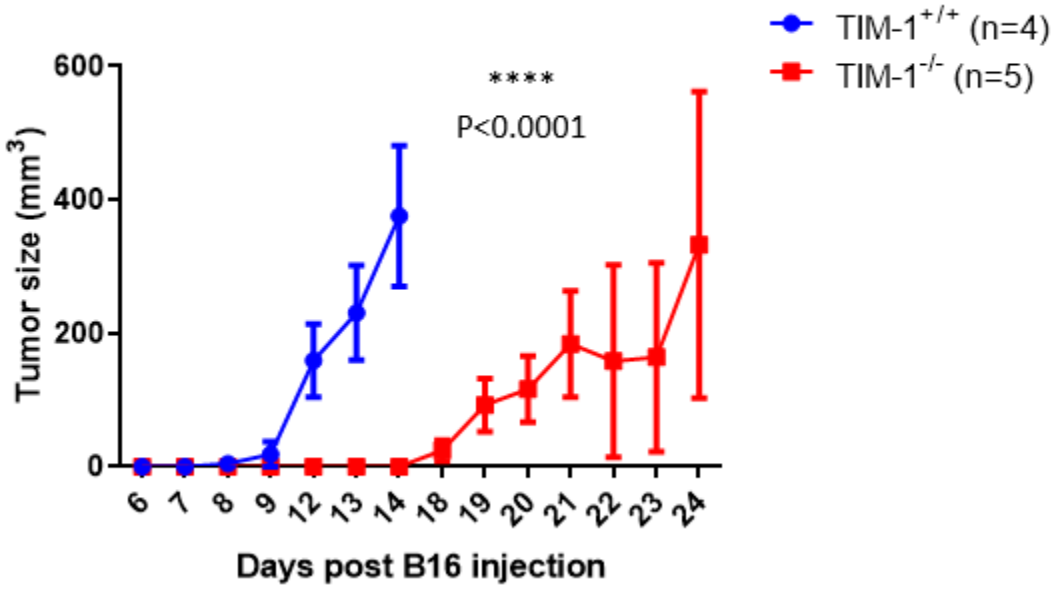
### 3.1 Delayed B16F1 melanoma tumour growth and improved survival in Tim-1-deficient mice

I aimed to investigate tumour growth and survival in Tim-1-deficient and Tim-1-expressing (wild-type) C57BL/6 mice using the syngeneic B16F1 melanoma cell line. Mice received a subcutaneous inoculation of $5x10^5$ B16F1 melanoma cells and were weighed, monitored for cachexia, and tumour growth was measured using calipers over time. While I initially hypothesized poorer outcomes in Tim-1-deficient mice, I observed improved survival and delayed tumour growth in Tim-1-deficient mice, compared to wild-type, Tim-1-expressing mice (Figure 1A and 1B). Upon repeating the experiment, I confirmed the original findings, as similar results were observed (Figure 1C and 1D). Tim-1-deficient mice exhibited improved survival and delayed tumour growth, compared to Tim-1-expressing mice (Figure 1C and 1D). Spider plots depicting tumour growth from individual mice are shown in Supplementary Figure 1A and 1B. These findings suggest that Tim-1 expression may promote tumour growth.

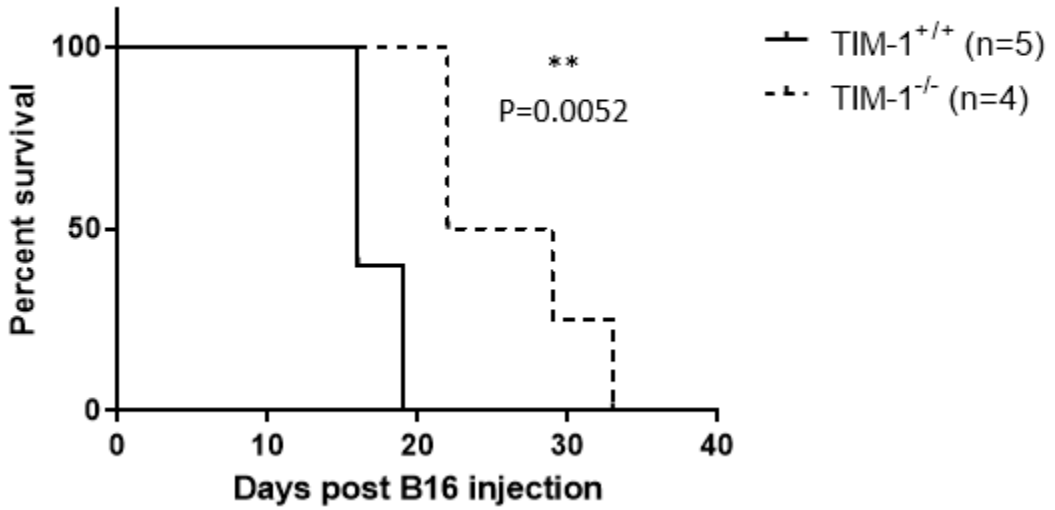
**A.**


Percent survival
100
50
0
0
10
20
30
Days post B16 injection
**
P=0.0047
TIM-1+/+ (n=4)
TIM-1-/- (n=5)

**B.**


Tumor size (mm3)
600
400
200
0
6
7
8
9
12
13
14
18
19
20
21
22
23
24
Days post B16 injection
****
P<0.0001
TIM-1+/+ (n=4)
TIM-1-/- (n=5)

**C.**




**D.**

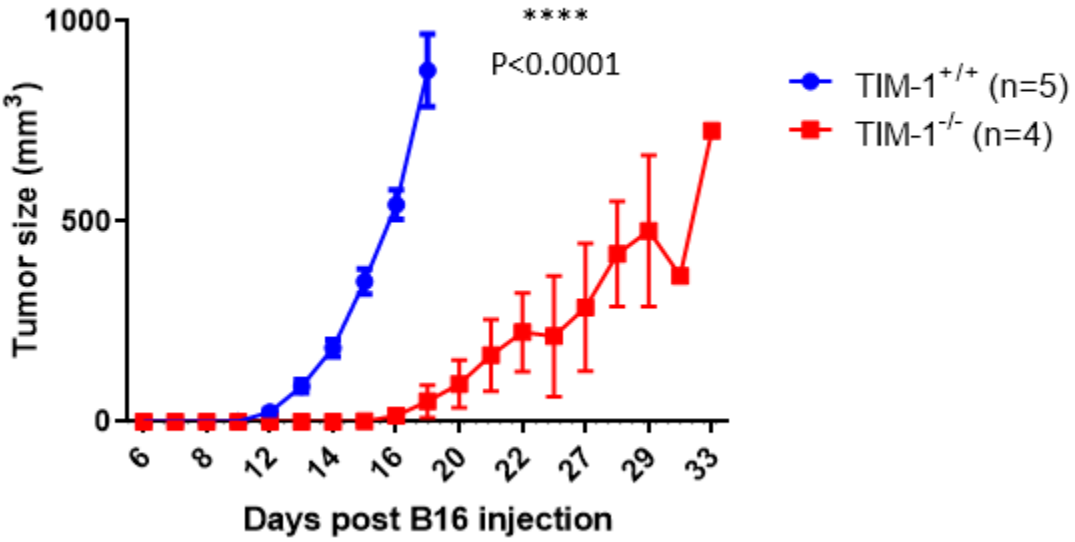


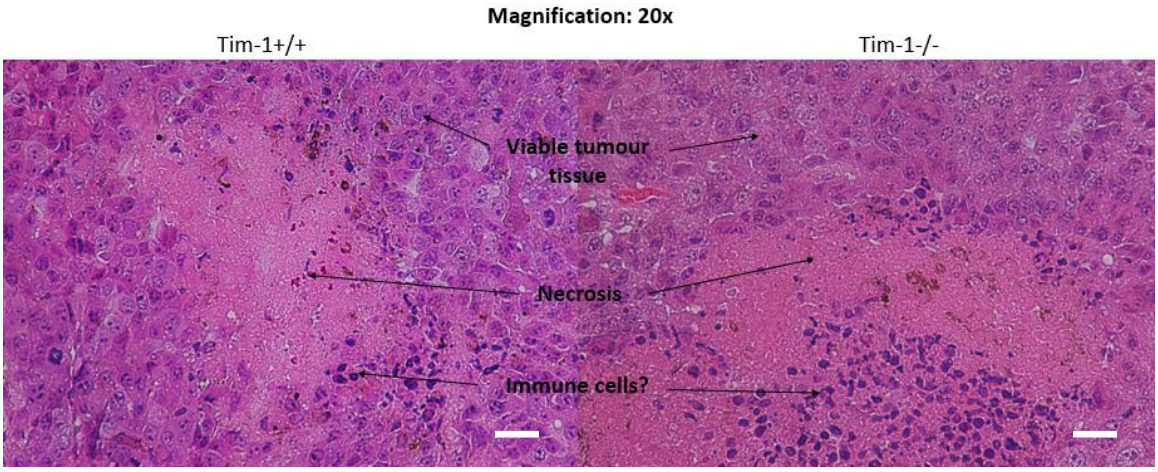

**Figure 1. Delayed B16F1 melanoma tumour growth and improved survival in immunocompetent Tim-1-deficient mice.**

Tim-1-expressing and Tim-1-deficient male C57BL/6 mice at 8-10 weeks old were subcutaneously inoculated with $5x10^5$ B16F1 melanoma. Endpoints were determined by incidence of cachexia, tumour ulceration, or a mean tumour diameter exceeding 10mm. **(A)** Survival curve (Mantel-Cox test, **p=0.0047) and **(B)** tumour size ($mm^3$) of inoculated Tim-1-expressing (n=4) and Tim-1-deficient (n=5) mice (Repeated Measures ANOVA, ****p<0.0001). In a repeated experiment, **(C)** survival curve (Mantel-Cox test, **p=0.0052) and **(D)** tumour size ($mm^3$) of inoculated Tim-1-expressing (n=5) and Tim-1-deficient (n=4) mice (Repeated Measures ANOVA, ****p<0.0001).

## 3.2 Reduced levels of tumour necrosis in Tim-1-deficient mice

Tumour necrosis in biopsy specimen from patients with melanoma is considered to be a poor prognostic factor [95]. To examine the levels of necrosis within the implanted B16F1 melanoma tumours, tumours were excised when they reached a diameter of ~10mm. The tumours were fixed in 10% formalin, embedded in paraffin, and sectioned before staining with H&E to observe necrosis. Healthy, viable tumour cells are stained purple with a large, visible nuclei and have a defined cell shape. In contrast, necrotic tumour cells are stained pink with a loss of nuclei [96-98]. Another population of cells with a darker nucleus was noted by the pathologist to potentially be infiltrating lymphocytes, which warrants further study to determine the phenotype of these cells using immunohistochemistry (Figure 2A). The percent necrosis was scored by a pathologist and it was observed that tumours in Tim-1-expressing mice exhibited greater levels of necrosis, compared to tumours in Tim-1-deficient mice (Figure 2B).

**A.**




**B.**

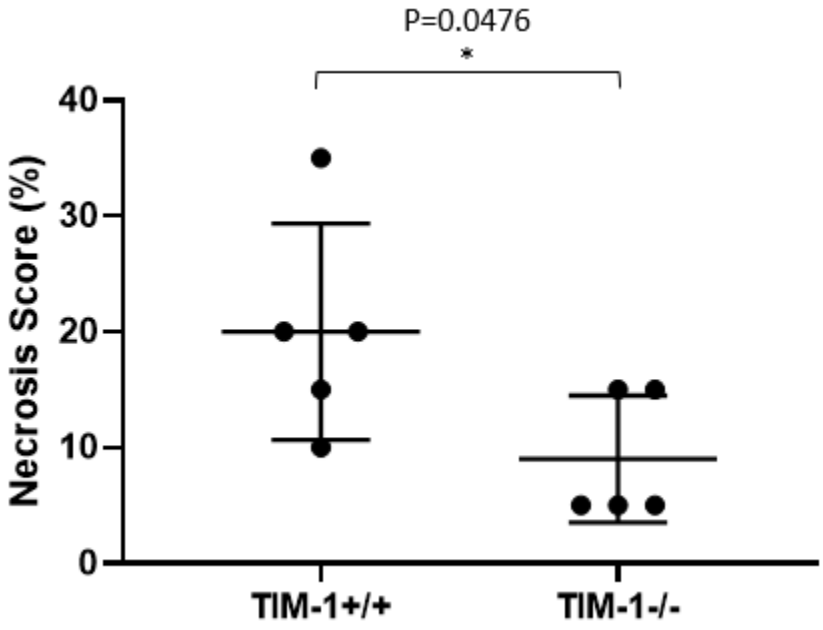



**Figure 2. Reduced necrosis in tumours of Tim-1-deficient mice.**

B16F1 melanoma tumours were excised from Tim-1-expressing and Tim-1-deficient mice at their endpoints. Tumours were fixed in 10% neutral buffered formalin and embedded in

paraffin before sectioning and staining with H&E. **(A)** Tumour sections from Tim-1-expressing and Tim-1-deficient mice are shown at 20x magnification. Scale bar = 100 microns. Areas of necrosis, viable tumour tissue, and suspected TILs are as labelled. **(B)** Percent necrosis as scored by a pathologist (Mann-Whitney, *p=0.0476).

## 3.3 *In vitro* T cell killing of B16F1 melanoma cells

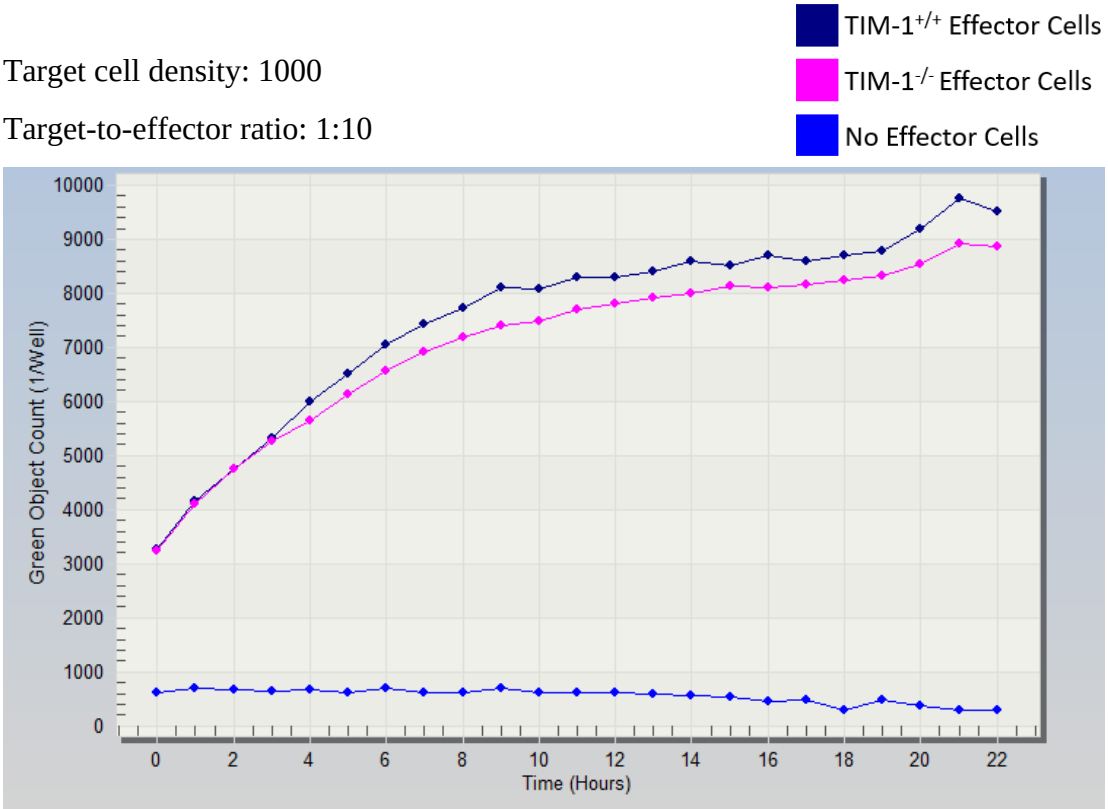
Using the IncuCyte Live Cell Imaging System, I sought to assess *in vitro* T cell killing of B16F1 melanoma cells. From tumour-bearing mice, I isolated Tim-1-deficient and Tim-1-expressing splenocytes as effector cells for coculture with the adherent B16F1 melanoma cell line. Graphs were generated using the IncuCyte software to depict the events of green fluorescence (ie. cell death) over time.

Although statistical analyses could not be performed due to the low number of replicates, there did not seem to be any differences in tumour cell death between cocultures using Tim-1-expressing or Tim-1-deficient effector cells (Figure 3A). However, upon analysis of the images taken by the IncuCyte software, I observed that both tumour cells and effector cells were undergoing cell death and took up the Sytox green nucleic acid stain (Figure 3B). This caused cell death between target and effector cells to be indistinguishable. In addition, some graphs generated by the IncuCyte software appeared inconsistent and erratic (Figure 3A). Therefore, the immune cell killing of tumour cells between Tim-1-expressing and Tim-1-deficient effector cells could not be assessed using the current methodology.

**A.**

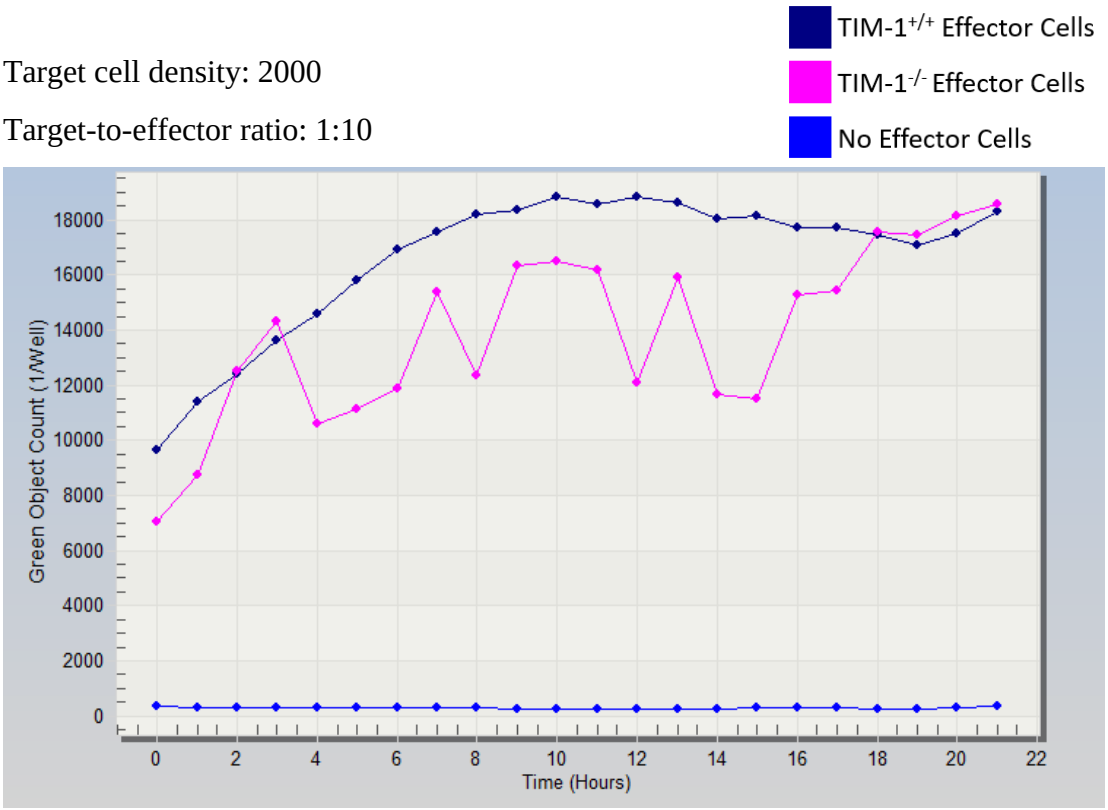
Target cell density: 1000

Target-to-effector ratio: 1:10



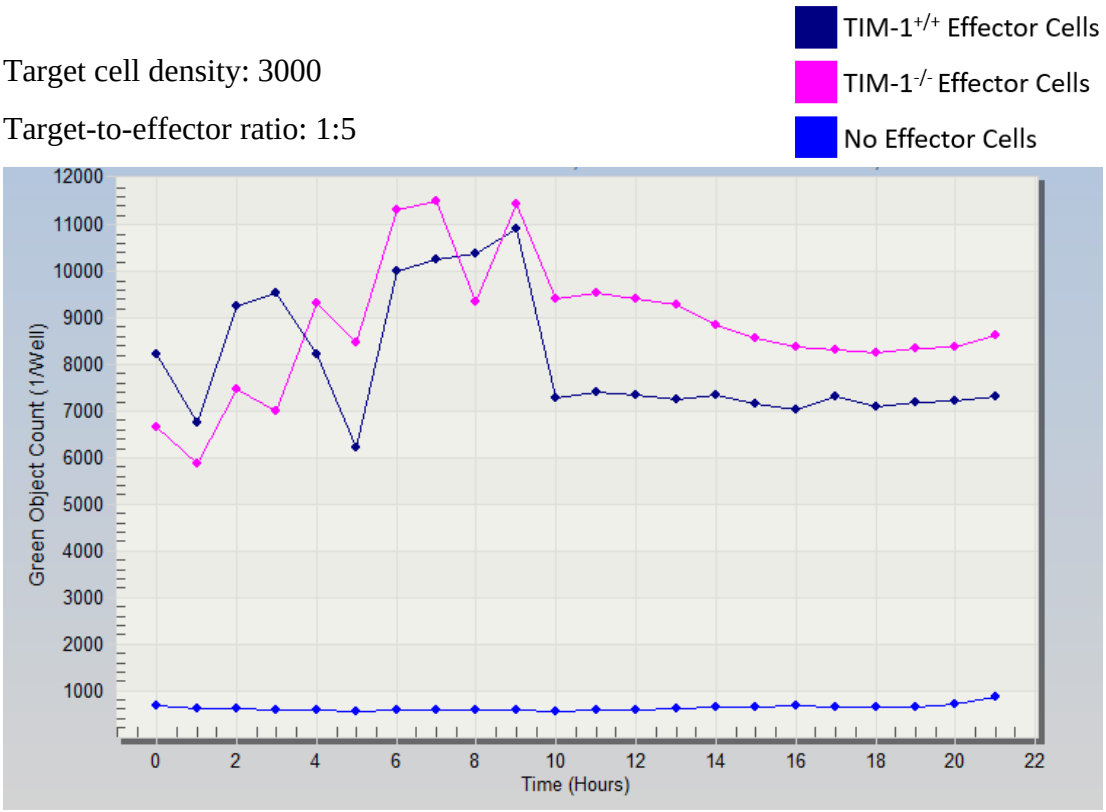

Target cell density: 2000

Target-to-effector ratio: 1:10



Target cell density: 3000

Target-to-effector ratio: 1:5




**B.**

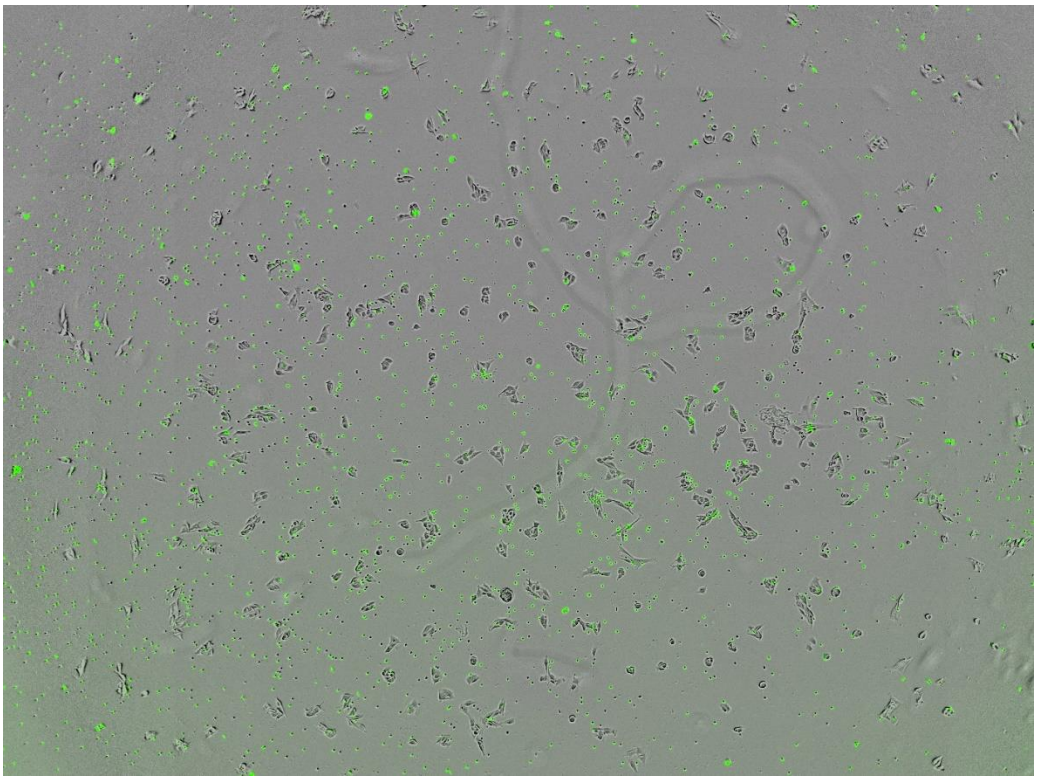


**Figure 3. IncuCyte immune cell killing assay.**

B16F1 melanoma target cells were seeded into a 96-well plate at various seeding densities (1000, 2000, and 3000 cells/well). Splenocytes were isolated from the spleens of tumour-bearing Tim-1-expressing and Tim-1-deficient C57BL/6 mice. Splenocytes were added to each well at various target to effector ratios (1:5 and 1:10). 100nm of Sytox green nucleic acid stain was added to each well to visualize cell death. Cocultures were incubated at 37ºC and 5% $CO_2$ for 24 hours, and images were taken hourly by the IncuCyte software. **(A)** Representative graphs of various cocultures generated by the IncuCyte software, depicting green fluorescent events (cell death) over time. **(B)** Representative image of a coculture taken by the IncuCyte software.

## 3.4 Decreased frequencies of Bregs in Tim-1-deficient mice immunized with B16F1 melanoma

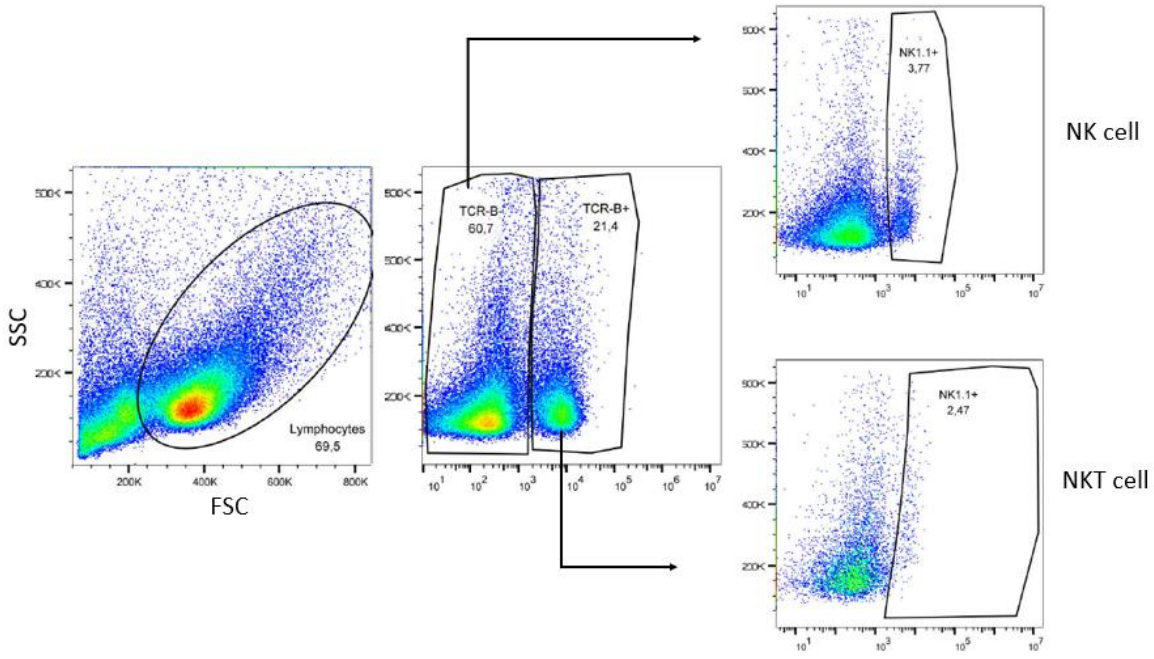
To obtain insight into the host's immune response elicited against B16F1 melanoma, I analyzed immune cell frequencies in the spleen of tumour-bearing mice. At their endpoints, the tumour-bearing mice were euthanized, and splenocytes were isolated and stained for multi-colour flow cytometric analysis. The gating strategy used and the analysis of the immune cells are shown in Figure 4A and 4B.

I examined the frequencies of CD8+ T cells, CD4+ T cells – Th1 cells, Th2 cells, and Tregs, NK cells, NKT cells, Bregs, and myeloid cells that include monocytic MDSCs and granulocytic MDSCs between Tim-1-expressing and Tim-1-deficient mice. Unexpectedly, I did not observe any differences in the frequency of these immune cell populations between Tim-1-expressing and Tim-1-deficient mice (Figure 4C).

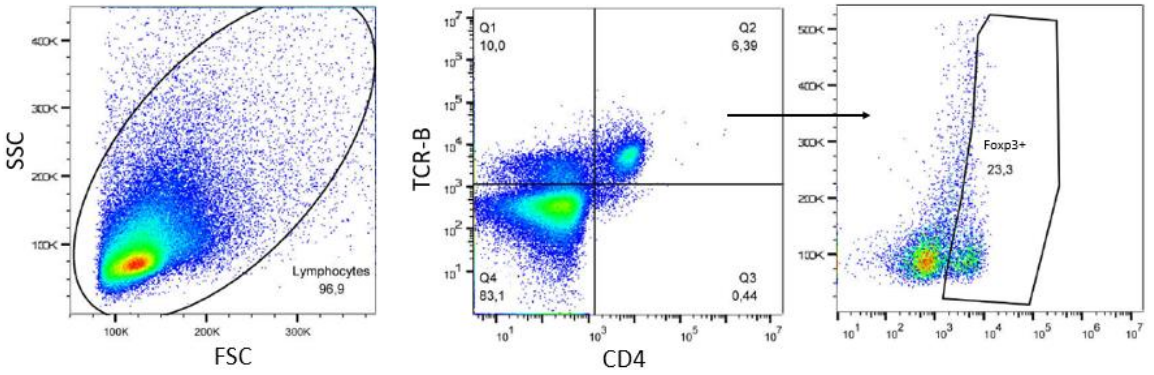
In order to stimulate an immune response against the tumour cells, I opted to immunize the Tim-1-expressing and Tim-1-deficient mice by subcutaneously injecting the mice with heat-killed (necrotic) B16F1 melanoma cells. Immunized mice were euthanized 7 days post-immunization, and splenocytes were isolated and stained for flow cytometric analysis as previously performed. I observed decreased frequencies of Bregs and myeloid cells with a phenotype consistent with monocytic MDSCs in the immunized Tim-1-deficient mice (Figure 4D). However, there were no significant differences in the frequency of other immune cell populations between the immunized Tim-1-expressing and Tim-1-deficient

mice (Figure 4D). These findings suggest that Tim-1 expression may promote tumour growth via increased frequencies of Bregs.

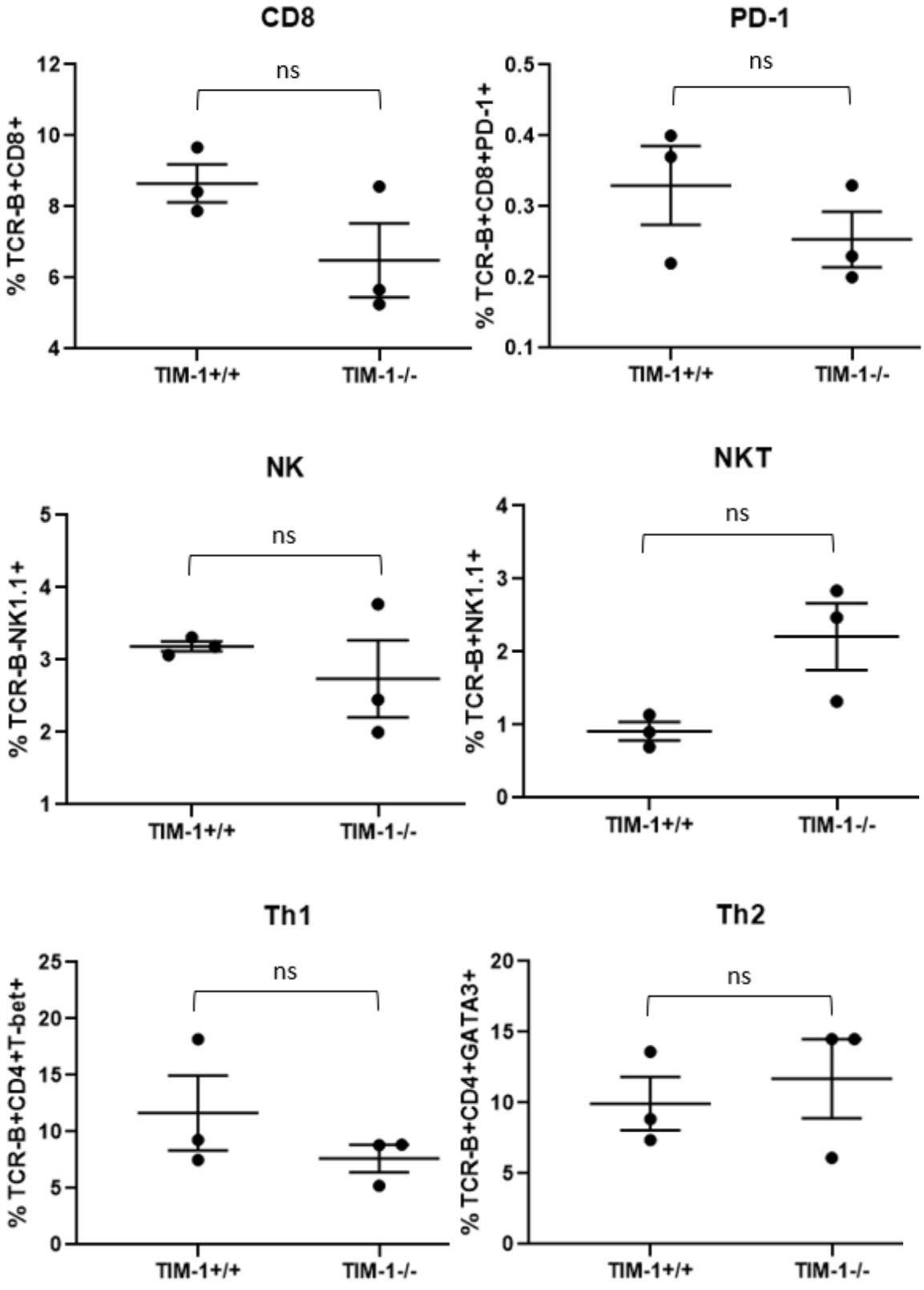
**A.**

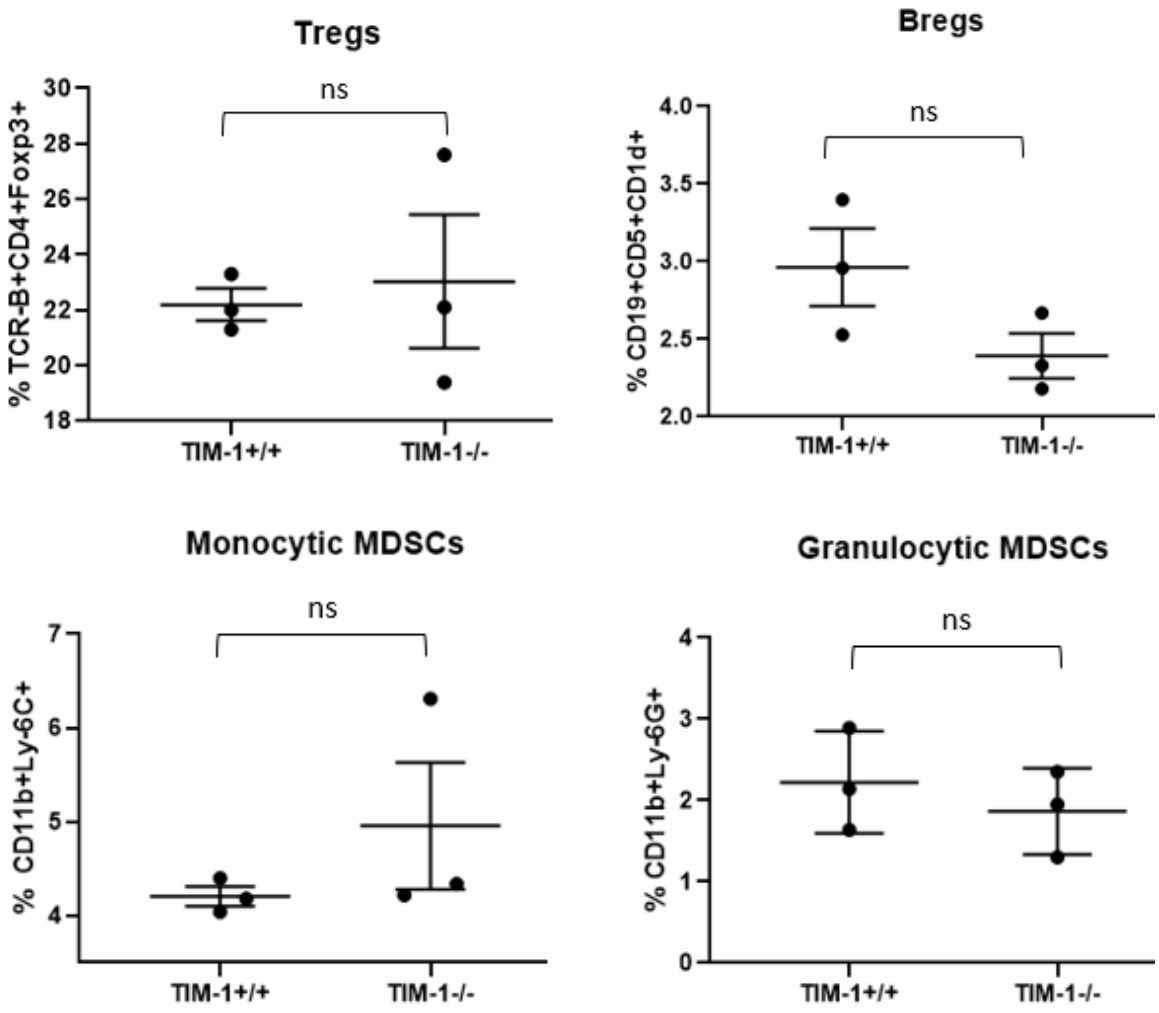

SSC
FSC
Lymphocytes
69,5
TCR-B-
60,7
TCR-B+
21,4
NK1.1+
3,77
NK cell
NK1.1+
2,47
NKT cell

**B.**

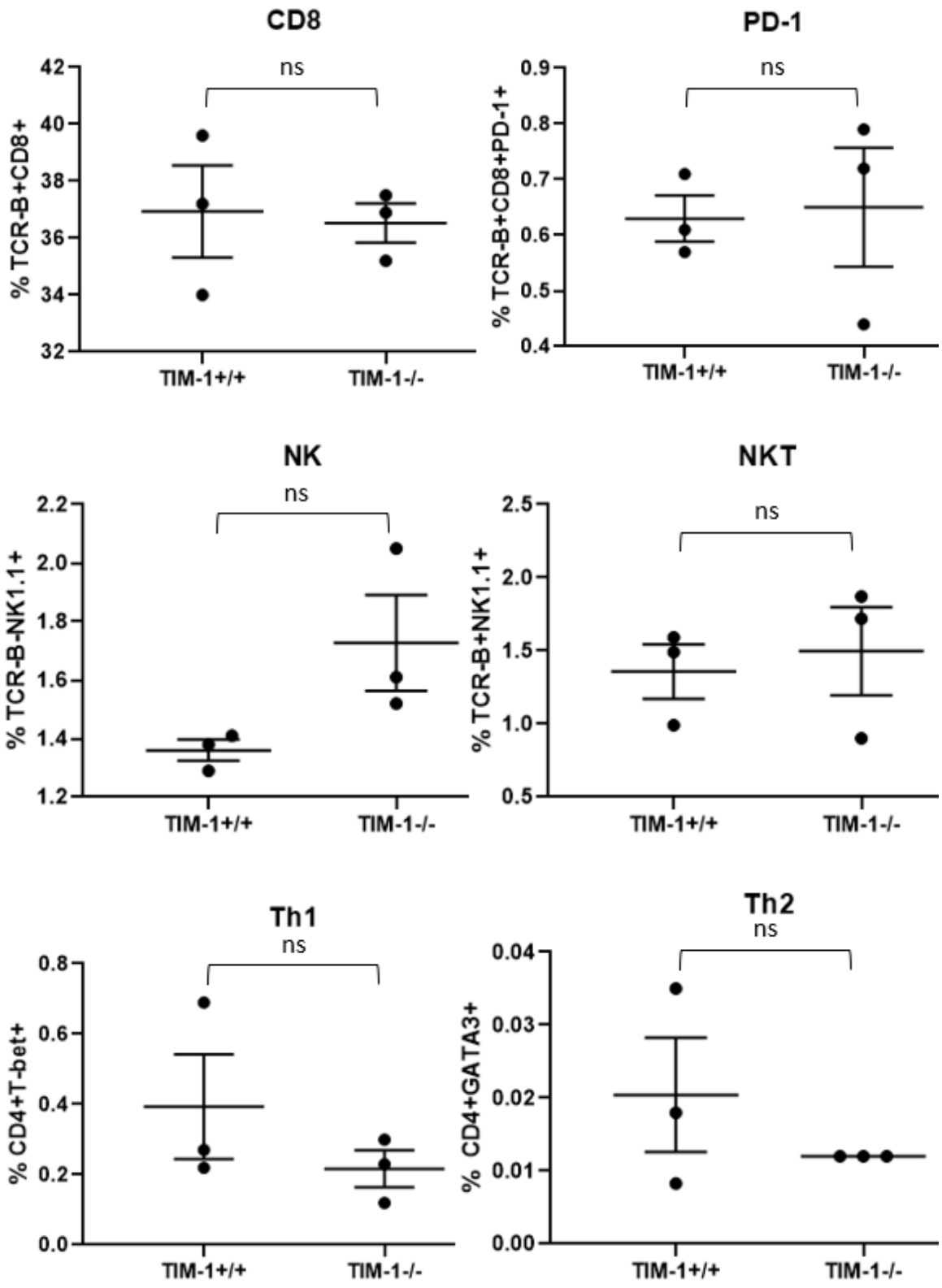

SSC
FSC
Lymphocytes
96,9
TCR-B
CD4
Q1
10,0
Q2
6,39
Q4
83,1
Q3
0,44
Foxp3+
23,3

**C.**


CD8
% TCR-B+CD8+
12
10
8
6
4
ns
TIM-1+/+
TIM-1-/-
PD-1
% TCR-B+CD8+PD-1+
0.5
0.4
0.3
0.2
0.1
ns
TIM-1+/+
TIM-1-/-
NK
% TCR-B-NK1.1+
5
4
3
2
1
ns
TIM-1+/+
TIM-1-/-
NKT
% TCR-B+NK1.1+
4
3
2
1
0
ns
TIM-1+/+
TIM-1-/-
Th1
% TCR-B+CD4+T-bet+
25
20
15
10
5
0
ns
TIM-1+/+
TIM-1-/-
Th2
% TCR-B+CD4+GATA3+
20
15
10
5
0
ns
TIM-1+/+
TIM-1-/-


Tregs
% TCR-B+CD4+Foxp3+
30
28
26
24
22
20
18
ns
TIM-1+/+
TIM-1-/-
Bregs
% CD19+CD5+CD1d+
4.0
3.5
3.0
2.5
2.0
ns
TIM-1+/+
TIM-1-/-
Monocytic MDSCs
% CD11b+Ly-6C+
7
6
5
4
ns
TIM-1+/+
TIM-1-/-
Granulocytic MDSCs
% CD11b+Ly-6G+
4
3
2
1
0
ns
TIM-1+/+
TIM-1-/-

**D.**


CD8
ns
% TCR-B+CD8+
42
40
38
36
34
32
TIM-1+/+
TIM-1-/-
PD-1
ns
% TCR-B+CD8+PD-1+
0.9
0.8
0.7
0.6
0.5
0.4
TIM-1+/+
TIM-1-/-
NK
ns
% TCR-B-NK1.1+
2.2
2.0
1.8
1.6
1.4
1.2
TIM-1+/+
TIM-1-/-
NKT
ns
% TCR-B+NK1.1+
2.5
2.0
1.5
1.0
0.5
TIM-1+/+
TIM-1-/-
Th1
ns
% CD4+T-bet+
0.8
0.6
0.4
0.2
0.0
TIM-1+/+
TIM-1-/-
Th2
ns
% CD4+GATA3+
0.04
0.03
0.02
0.01
0.00
TIM-1+/+
TIM-1-/-

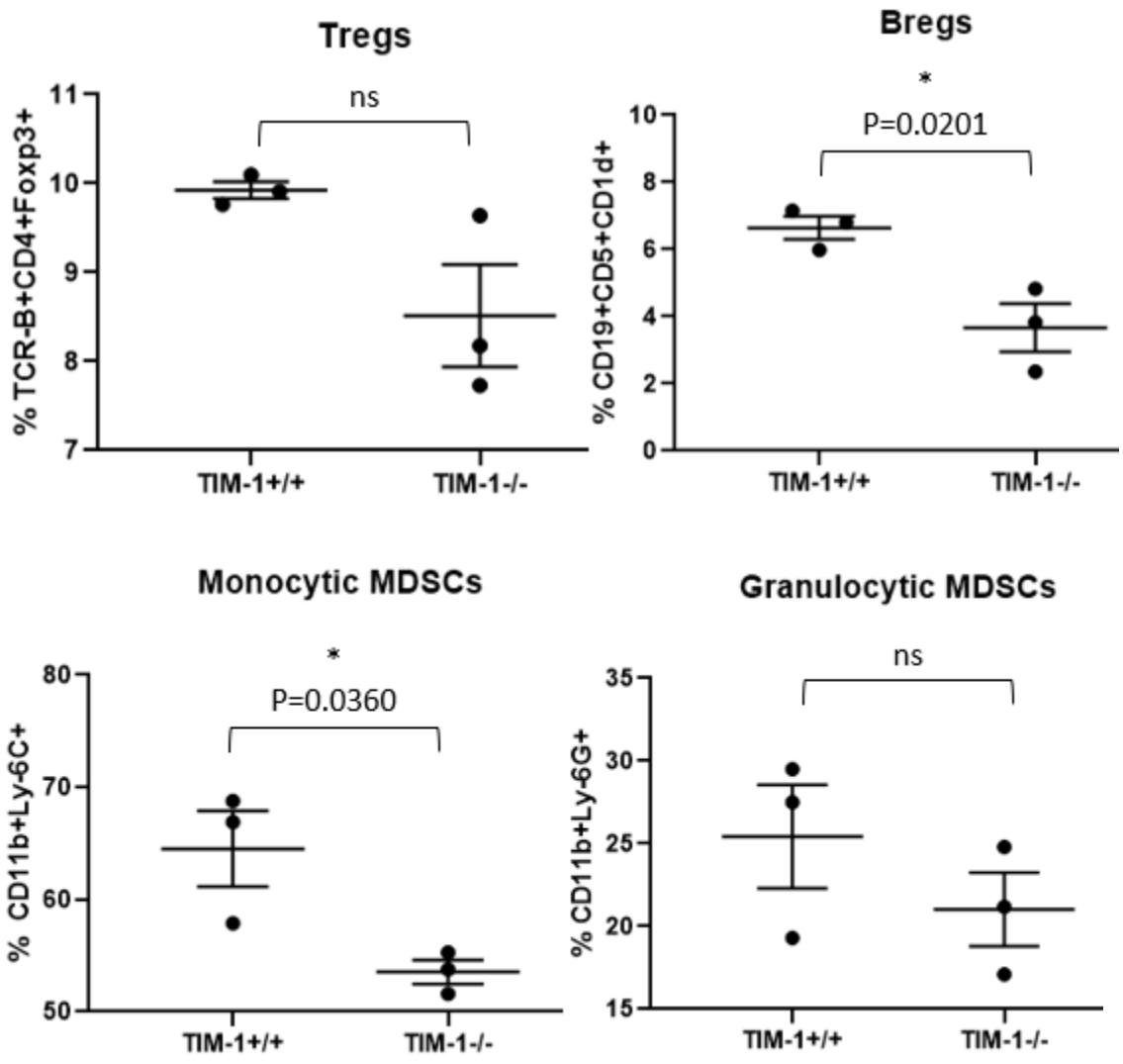



**Figure 4. Decreased frequencies of Bregs in Tim-1-deficient mice immunized with necrotic B16F1 melanoma cells.**

Splenocytes were isolated from B16F1 melanoma tumour-bearing Tim-1-expressing and Tim-1-deficient mice at their endpoints. Splenocytes were stained with fluorochrome-conjugated antibodies for analysis using flow cytometry. **(A)** Gating strategy for extracellular staining of NK cells and NKT cells. **(B)** Gating strategy for intracellular

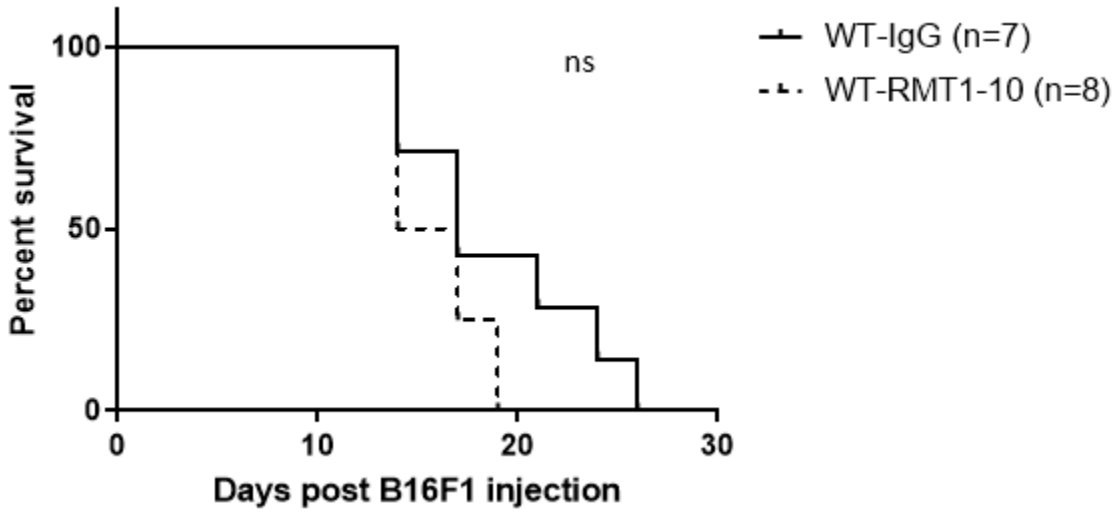
staining of Tregs. **(C)** Frequency of immune cell populations in splenocytes of Tim-1-expressing (n=3) and Tim-1-deficient (n=3) mice (unpaired t-test). To induce an immune response, Tim-1-expressing and Tim-1-deficient mice were subcutaneously immunized with heat-killed B16F1 melanoma, and splenocytes were isolated 7 days post-immunization. **(D)** Frequency of immune cell populations in splenocytes of immunized Tim-1-expressing (n=3) and Tim-1-deficient (n=3) mice (unpaired t-test, *$p<0.05$).

## 3.5 Effect of anti-Tim-1 monoclonal antibody, RMT1-10, on immune responses against B16F1 melanoma

To test the effects of Tim-1 signalling in anti-tumour immune responses *in vivo*, I used the anti-Tim-1 mAb RMT1-10 to activate Tim-1 on immune cells. RMT1-10 is a low affinity anti-Tim-1 mAb that has been shown to induce the expansion of Tim-1-expressing Bregs [87]. RMT1-10 has been used in different murine models to reduce the severity of experimental autoimmune encephalomyelitis [99] and promote cardiac allograft tolerance at the doses used [100]. These data taken together further support the claim that RMT1-10 promotes Breg production.

I administered 300ug of RMT1-10 or 300ug of the isotype control intraperitoneally to Tim-1-expressing mice. Subsequently, mice were subcutaneously inoculated with $5x10^5$ B16F1 melanoma cells. I continued to administer either 300ug of RMT1-10 or 300ug of the isotype control to the respective mice every 3 days subsequent to tumour inoculation. I observed a slight trend of earlier tumour growth and poorer survival in mice receiving RMT1-10, compared to the mice receiving the isotype control (Figure 5A and 5B). Spider plots depicting tumour growth from individual mice are shown in Appendix B. However, these findings were not statistically significant. Further studies with an increased sample size in each group would be necessary to establish if this trend is statistically significant.

**A.**




**B.**

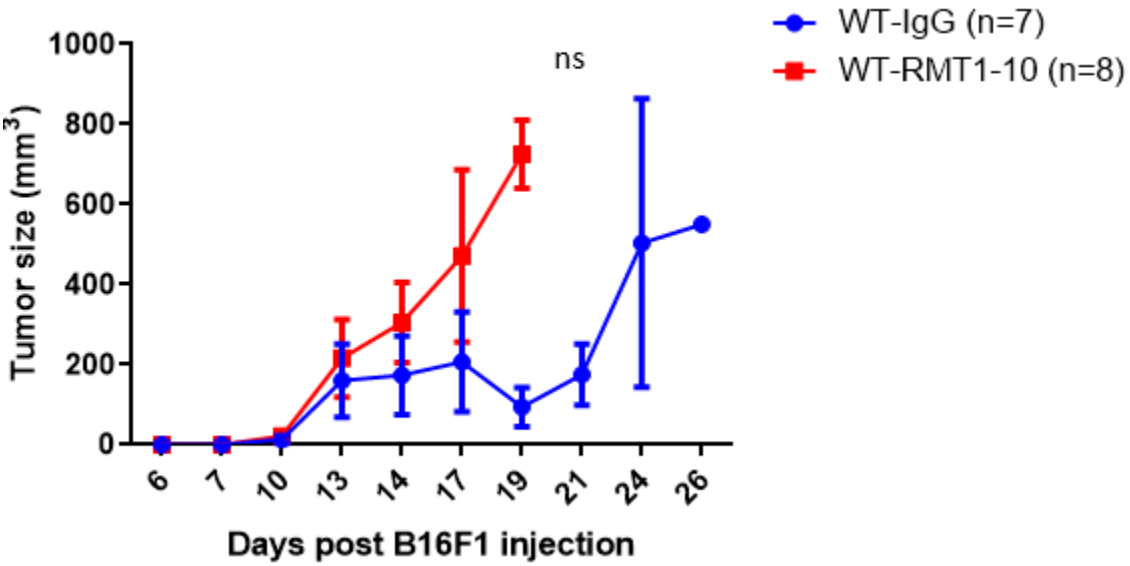



**Figure 5. Effect of anti-Tim-1 monoclonal antibody, RMT1-10, on B16F1 melanoma tumour growth and survival in Tim-1-expressing mice.**

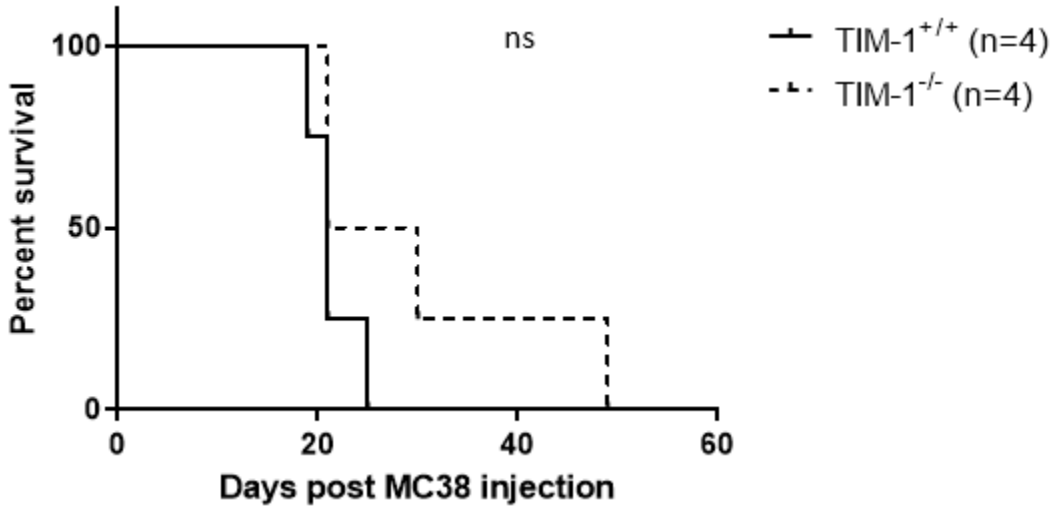
Tim-1-expressing mice received either 300ug of RMT1-10 (n=8) or 300ug of IgG control (n=7) intraperitoneally on days -1, 0, and every 3 days relative to subcutaneous B16F1

melanoma administration. Endpoints were determined by incidence of cachexia, tumour ulceration, or a mean tumour diameter exceeding 10mm. **(A)** Percent survival (Mantel-Cox test) and **(B)** tumour size ($mm^3$) of inoculated Tim-1-expressing mice treated with either RMT1-10 or IgG control (Repeated Measures ANOVA).

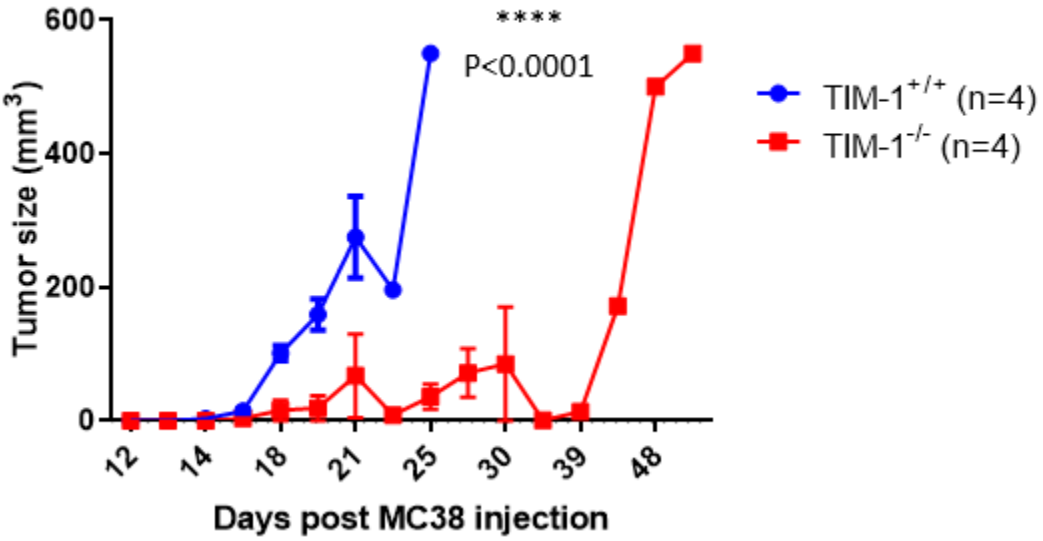
## 3.6 Effect of MC38 colorectal adenocarcinoma in Tim-1-expressing and Tim-1-deficient mice

Finally, in order to determine if the delayed tumour growth and improved survival observed in Tim-1-deficient mice inoculated with B16F1 melanoma were generalizable to other tumour cell lines, I tested tumour growth and survival using MC38 colorectal adenocarcinoma. Similar to the B16F1 melanoma cell line, MC38 colorectal adenocarcinoma is syngeneic to C57BL/6 mice. Tim-1-expressing and Tim-1-deficient mice were anesthetized using isoflurane and subcutaneously inoculated with $1x10^5$ MC38 colorectal adenocarcinoma cells. Consistent with the findings using B16F1 melanoma, Tim-1-deficient mice exhibited delayed tumour growth (Figure 6A). There were no significant differences in the survival between Tim-1-expressing and Tim-1-deficient mice (Figure 6B). However, when this experiment was repeated with additional mice in each group, no significant differences were observed in tumour growth and survival (Figure 6C and 6D). Spider plots depicting tumour growth from individual mice are shown in Supplementary Figure 3A and 3B. This potentially indicates that the effects of Tim-1 on anti-tumour immune response may be tumour-dependent.

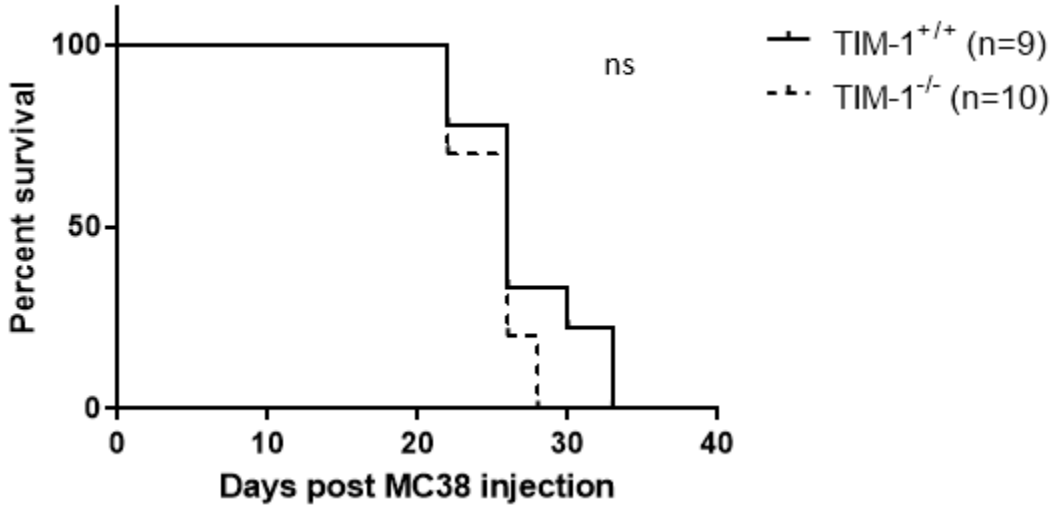
**A.**


ns
TIM-1+/+ (n=4)
TIM-1-/- (n=4)
Percent survival
100
50
0
0
20
40
60
Days post MC38 injection

**B.**


****
P<0.0001
TIM-1+/+ (n=4)
TIM-1-/- (n=4)
Tumor size (mm3)
600
400
200
0
12
14
18
21
25
30
39
48
Days post MC38 injection

**C.**




**D.**

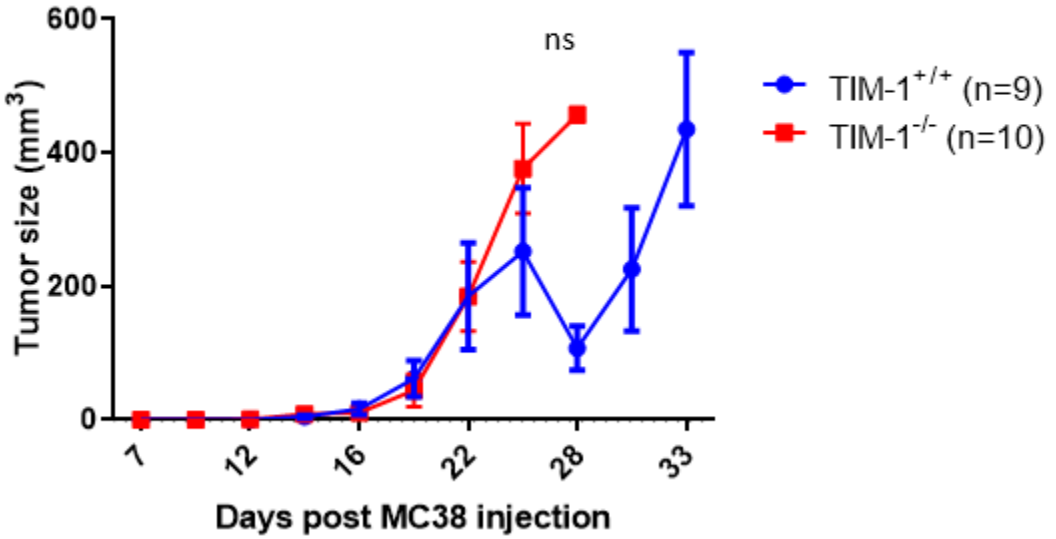



**Figure 6. Effect of MC38 colorectal adenocarcinoma on Tim-1-expressing and Tim-1-deficient mice.**

Tim-1-expressing and Tim-1-deficient male C57BL/6 mice at 8-10 weeks old were subcutaneously inoculated with $1x10^5$ MC38 colorectal adenocarcinoma. Endpoints were

determined by incidence of cachexia, tumour ulceration, or a mean tumour diameter exceeding 10mm. **(A)** Percent survival (Mantel-Cox test) and **(B)** tumour size ($mm^3$) of inoculated Tim-1-expressing (n=4) and Tim-1-deficient (n=4) mice (Repeated Measures ANOVA, ****$p<0.0001$). In a repeated experiment, **(C)** percent survival (Mantel-Cox test) and **(D)** tumour size ($mm^3$) of inoculated Tim-1-expressing (n=9) and Tim-1-deficient (n=10) mice (Repeated Measures ANOVA).

# Chapter 4

# 4 DISCUSSION

## 4.1 Overview of study

Tim-1 is a cell-surface glycoprotein expressed on a variety of immune cells. Tim-1-expressing immune cells are implicated in allergy, asthma, autoimmune disease, and transplant tolerance. Through different mechanisms, Tim-1 can promote immune responses through T cells and iNKT cells or inhibit immune responses through Breg function [54-93]. However, the role of Tim-1 in anti-tumour immune responses has not been studied in detail.

In this study, I examined the anti-cancer effects of Tim-1 in two mouse tumour models. I initially hypothesized that Tim-1-expressing mice would have an improved prognosis due to the induction of proinflammatory responses of CD4+ T cells and iNKT cells induce by Tim-1. Surprisingly, my findings have demonstrated that Tim-1-deficient mice exhibited delayed tumour growth and improved survival, compared to Tim-1-expressing mice. These findings were accompanied by reduced necrosis in the tumours of Tim-1-deficient mice, which is an indicator of improved prognosis in human melanoma [95]. In addition, reduced frequencies of Bregs were found in B16F1 melanoma-immunized Tim-1-deficient mice. These results suggest that Tim-1 expression in the immune system may be detrimental to anti-tumour immunity, potentially by inducing Bregs. Accordingly, I have revised my hypothesis, and I propose that Tim-1-expressing Bregs contribute to the inhibition of anti-tumour immune responses against B16F1 melanoma.

In my immune cell killing assay using the IncuCyte Live Cell Imaging System, graphs generated by the IncuCyte software appeared inconsistent and erratic. Additionally, I observed cell death in both the tumour cells and effector cells, and cell death between the target and effector cells were indistinguishable. Thus, I was unable to compare the immune cell killing of tumour cells between Tim-1-expressing and Tim-1-deficient effector cells. The unexpected issue of effector cells undergoing cell death and taking up the nucleic acid stain potentially contributed to the inaccuracies of the graphs, but this can be rectified by further isolating T cells from splenocytes for use as effector cells and by implementing a red cytoplasmic dye to stain the target cells. These changes will ensure the ability to distinguish cell death between the target and effector cells, and potentially demonstrate reduced *in vitro* killing of B16F1 melanoma cells by T cells from Tim-1-expressing mice.

Finally, when Tim-1-expressing and Tim-1-deficient mice were inoculated with syngeneic MC38 colorectal adenocarcinoma, I observed delayed tumour growth in the Tim-1-deficient mice, while there were no significant differences in the survival between Tim-1-expressing and Tim-1-deficient mice. Unexpectedly, when this experiment was repeated, there were no significant differences in tumour growth or survival. Due to the inconsistencies of these data, it will be necessary to repeat this experiment to confirm my findings. Nevertheless, this potentially suggests that the role of Tim-1 in anti-tumour immunity is a phenomenon that may be restricted to certain types of tumours.

## 4.2 Immune profiles of B16F1 melanoma and MC38 colorectal adenocarcinoma

Since I observed inconsistent findings using B16F1 melanoma and MC38 colorectal adenocarcinoma, I postulated that the effects of Tim-1 on anti-tumour immunity may differ depending on the type of tumour and the immune response elicited.

B16F1 melanoma was spontaneously derived, exhibits poor immunogenicity with low levels of immune cell infiltrates, and reduced susceptibility to cytotoxic T cell killing. This is due to its lack of MHC class I expression and chemokine production to recruit T cells and other immune cells [101, 102]. B16F10 melanoma cells, a highly metastatic B16 melanoma cell line, expresses high levels of β-catenin and low levels of other chemokines [103]. In human melanoma, β-catenin signalling is correlated to the prevention of T cell infiltration into tumours and resistance to immune checkpoint inhibition therapy [104]. However, it is important to note that human melanoma is highly immunogenic [102, 105]. In addition, a study showed that B16 melanoma cells express high levels of CTLA-4, which is an inhibitory ligand to suppress the function of T cells. It was also shown that B16 melanoma models were particularly resistant to immunotherapies, in contrast to other tumour models (eg. CT26 colon carcinoma, RENCA renal cell carcinoma, 4T1 mammary carcinoma) [102].

MC38 colorectal adenocarcinoma is a carcinogen-induced murine tumour cell line and is moderately immunogenic [106]. Immune cell infiltrates within MC38 tumours in mice include CD8+ T cells and NK cells, with gene expression signatures suggesting that cytolytic activity occurs [106, 107]. However, immunosuppressive cells, such as Tregs and MDSCs, were also abundant [106, 107]. This data is further supported by the detection of

the chemokine, KC/GRO, which recruit MDSCs and neutrophils to the tumour [106]. In response to checkpoint inhibition therapies, the MC38 colorectal adenocarcinoma model was modestly responsive to checkpoint inhibition, in comparison to other highly responsive murine colon cancers (eg. CT26 colon carcinoma) [107].

Due to the differences between B16F1 melanoma and MC38 colorectal adenocarcinoma, I speculate that the immune responses influenced by Tim-1 on these tumours may be dissimilar.

## 4.3 Potential role of Tim-1-expressing Bregs in suppressing anti-tumour immunity

My findings suggest that Tim-1 expression on Bregs may potentially impede anti-tumour immunity. Bregs secrete IL-10 and TGF-$\beta$, cytokines that suppress proinflammatory cells and promote the development and recruitment of Tregs, consequently suppressing anti-tumour immune responses [31, 32]. In a study of murine breast cancer, it was demonstrated that tumour-evoked Bregs promote tumour growth and lung metastases [108]. This effect was indicated to be a result of Treg conversion from resting CD4+ T cells [32, 108]. In addition, tumour-evoked Bregs have been shown to play an important role in regulating the ability of MDSCs to promote metastases and immunosuppression [109]. This is consistent with my findings, where decreased frequencies of both Bregs and potential monocytic MDSCs were observed in the spleens of B16F1 melanoma-immunized Tim-1-deficient mice.

Since Tim-1 signalling induces Breg production/activation and Tim-1 is expressed by ~70% of IL-10 producing Bregs [87], a possible therapeutic would aim to block Tim-1

signalling. My future objective would be to generate a monoclonal antibody to target Tim-1 and inhibit activation/signalling. This therapeutic strategy could potentially reduce the immunosuppressive activity of Bregs and improve anti-cancer immune responses.

## 4.4 Future studies

Since I have established that Tim-1-deficient mice have a better prognosis against B16F1 melanoma than Tim-1-expressing mice, it is necessary to determine if Tim-1-expressing Bregs are inhibiting anti-tumour immune responses. To achieve this, my next step would be to perform an adoptive transfer using isolated B cells from naive Tim-1-expressing mice, tumour-bearing Tim-1-expressing mice, or tumour-bearing Tim-1-deficient mice into naïve Tim-1-deficient recipients before tumour inoculation. I expect Tim-1-deficient recipients of Tim-1-expressing B cells will have accelerated tumour growth and decreased survival compared to Tim-1-deficient recipients of Tim-1-deficient B cells.

To improve the *in vitro* T cell killing assay using the IncuCyte system, I would incorporate a Red IncuCyte CytoLight Rapid Dye (Sartorius) to selectively label target tumour cells. This will allow us to pinpoint cell death specifically on the target cells and exclude the effector lymphocytes.

Alternatively, I can utilize MHC tetramers to analyze the antigen (tumour)-specific T cell response *in vitro.* MHC tetramers are complexes of 4 MHC molecules which are associated with a specific peptide and are bound to a fluorochrome for analysis using flow cytometry. I will inoculate Tim-1-expressing and Tim-1-deficient mice with B16F10 melanoma expressing the artificial neoantigen, ovalbumin, which will induce a T cell response against the tumour. Using MHC tetramers with an ovalbumin peptide, I can isolate splenocytes

from the tumour-inoculated mice and detect antigen (ovalbumin)-specific CD8+ cytotoxic T cells.

## 4.5 Limitations

My study has a number of limitations. Due to the nature of this study, I required the use of Tim-1-expressing (wild-type) and Tim-1-deficient (Tim-$1^{-/-}$) mice. Currently, the Gunaratnam laboratory only has access to this model on a C57BL/6 genetic background, which limits the tumour models to cell lines derived from C57BL/6 mice. In future studies, I plan to test tumour models syngeneic to BALB/c mice, which will expand the types of cancers that can be examined and confirm my findings in different strains of mice.

It has been shown that C57BL/6 mice are predisposed to developing a Th1 immune response, while BALB/c mice preferentially develop a Th2 immune response [114]. Since Tim-1 is preferentially expressed by Th2 cells over Th1 cells, my results may not accurately represent the effects of Tim-1 on host lymphocytes in anti-tumour immunity. However, this can be better examined when tumour models in Tim-1-expressing and Tim-1-deficient BALB/c mice are implemented in the future.

The mouse models used in this study were restricted to male mice, which do not accurately represent or account for the sex differences observed in the respective cancer. In the United States, the incidence and mortality rate of cancer were higher in men than women from 2009 to 2013 [110]. Additionally, the differences in sex hormones between men and women (eg. testosterone and estrogen, respectively) can impact the development of certain cancers [111]. Studies have shown that historically, males are predominantly used in clinical trials, while females were excluded [112], and increased adverse drug reactions against anti-cancer therapeutics were observed more often in women than men [113]. As a

result, it is important to take the effect of sex differences in cancer into consideration and include studies using both males and females.

In this study, I demonstrated that Tim-1-deficient mice immunized with heat-killed B16F1 melanoma cells exhibited decreased frequencies of splenic Bregs and a myeloid population with potential monocytic MDSCs. While the cell-surface markers used in this study to identify monocytic MDSCs (CD11b+, Ly-6C+) provide an initial framework for characterizing MDSCs, additional markers can be implemented to characterize these cells in further detail [115]. To identify monocytic MDSCs, additional cell-surface markers including Gr-$1^{mid}$ and CD49d+ can be used [116], and additional markers to identify granulocytic MDSCs in further detail include Gr-$1^{hi}$ [116].

In addition, the immune cells I have characterized in this study were restricted to splenocytes. In order to accurately examine anti-tumour immune responses, infiltrating lymphocytes into the tumour and immune cells in the tumour-draining lymph nodes should be examined. Moreover, only three mice were used to analyze the splenic immune cell frequencies between Tim-1-expressing and Tim-1-deficient mice, which may not be sufficient to confidently draw firm conclusions. Therefore, it will be necessary to perform additional experiments to investigate the populations of immune cells present in the tumour and lymph nodes of tumour-bearing and tumour-immunized Tim-1-expressing and Tim-1-deficient mice.

Lastly, while subcutaneous tumours can be easily observed and measured with minimal invasiveness, research has shown that the site of tumour inoculation can affect anti-tumour immune responses. In a study of immunotherapies for murine colon cancer, the immune microenvironment of a subcutaneous tumour differed significantly from an orthotopic

gastrointestinal tumour [117]. While the establishment of an orthotopic tumour graft via surgical procedure gives rise to excessive inflammation, these researchers developed a novel orthotopic model using endoscopy-guided microinjection of tumour cells to reduce inflammation. They found that the immune microenvironment of the subcutaneous tumour graft was comprised of lower levels of immune cell infiltrates in the tumour and increased immunosuppressive cells compared to the orthotopic graft [117]. However, for the purposes of my study and for the sake of simplicity, I focused on elucidating the effects of Tim-1 on immune responses against tumours. Despite using the subcutaneous injection model, comparisons of immune responses between Tim-1-expressing and Tim-1-deficient mice can still be made. Once I establish the role of Tim-1 in anti-tumour immunity and specific mechanisms of Tim-1 function, I can address any discrepancies between subcutaneous and orthotopic tumour administration in future studies.

## 4.6 Conclusion

Cancer remains a highly prevalent and deadly disorder. Although, novel immunotherapies and checkpoint inhibitors have shown some success in patients, only a fraction of patients respond to these therapies. In this study, my work has demonstrated that Tim-1-deficient mice have improved outcomes when inoculated with B16F1 melanoma tumours, compared to Tim-1-expressing mice. Tim-1-deficient mice exhibited improved survival, delayed tumour growth, reduced tumour necrosis, and reduced frequencies of splenic Bregs. With additional research, these findings may potentially lead to improved anti-cancer treatments aimed at suppressing the function of Tim-1-expressing Bregs.

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# APPENDICES

## Appendix A. Animal Ethics Approval

Western

**AUP Number: 2018-147**
**PI Name: Gunaratnam, Lakshman**
**AUP Title: Establishing the Role of Kidney Injury Molecule-1 (KIM-1) in Cancer**
**Official Notification of ACC Approval:** A MODIFICATION to Animal Use Protocol **2018-147** has been approved.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within thi s AUP.

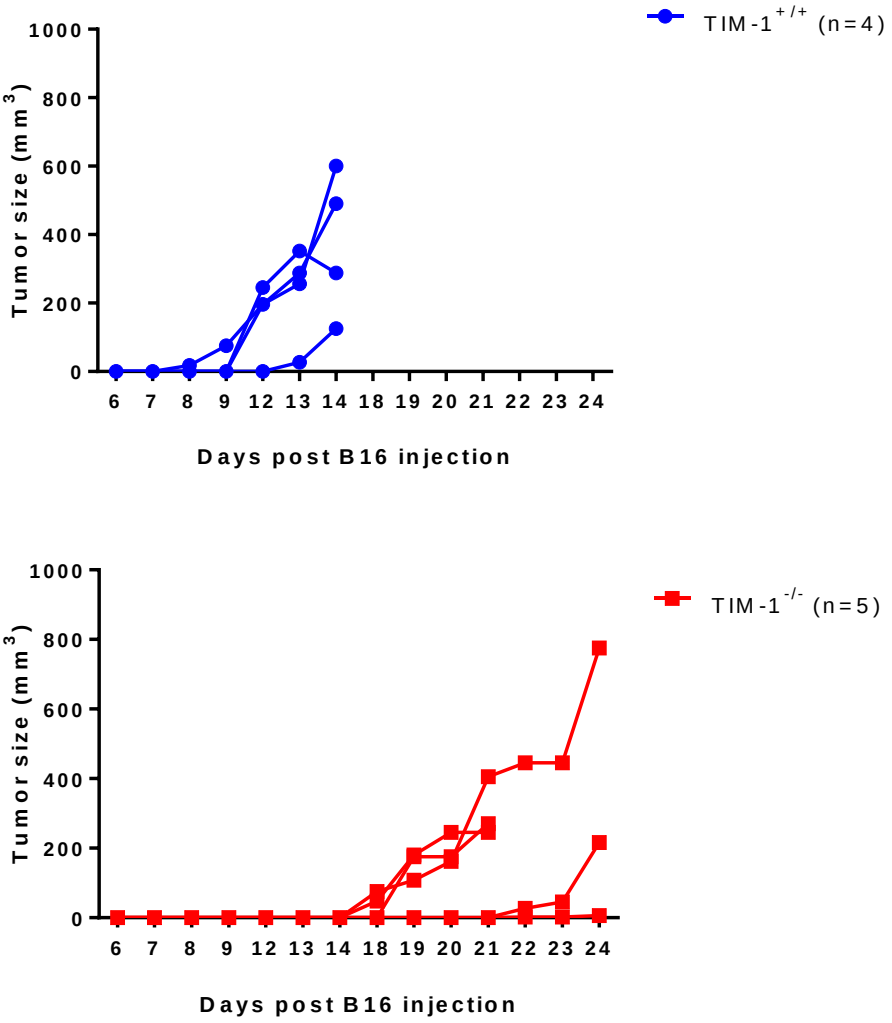
As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15 http://www.uwo.ca/univsec/policies_procedures/research.html
   b) University Council on Animal Care Policies and related Animal Care Committee procedures
   c) http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm
2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.http://uwo.ca/research/services/animalethics/animal_use_protocols.html
3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training ([training@uwo.ca]training@uwo.ca ); and
   c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:
      i) Research Animal Procurement
      ii) Animal Care and Use Records
      iii) Sick Animal Response
      iv) Continuing Care Visits
5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance
   the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, http://www.uwo.ca/hr/learning/required/index.html

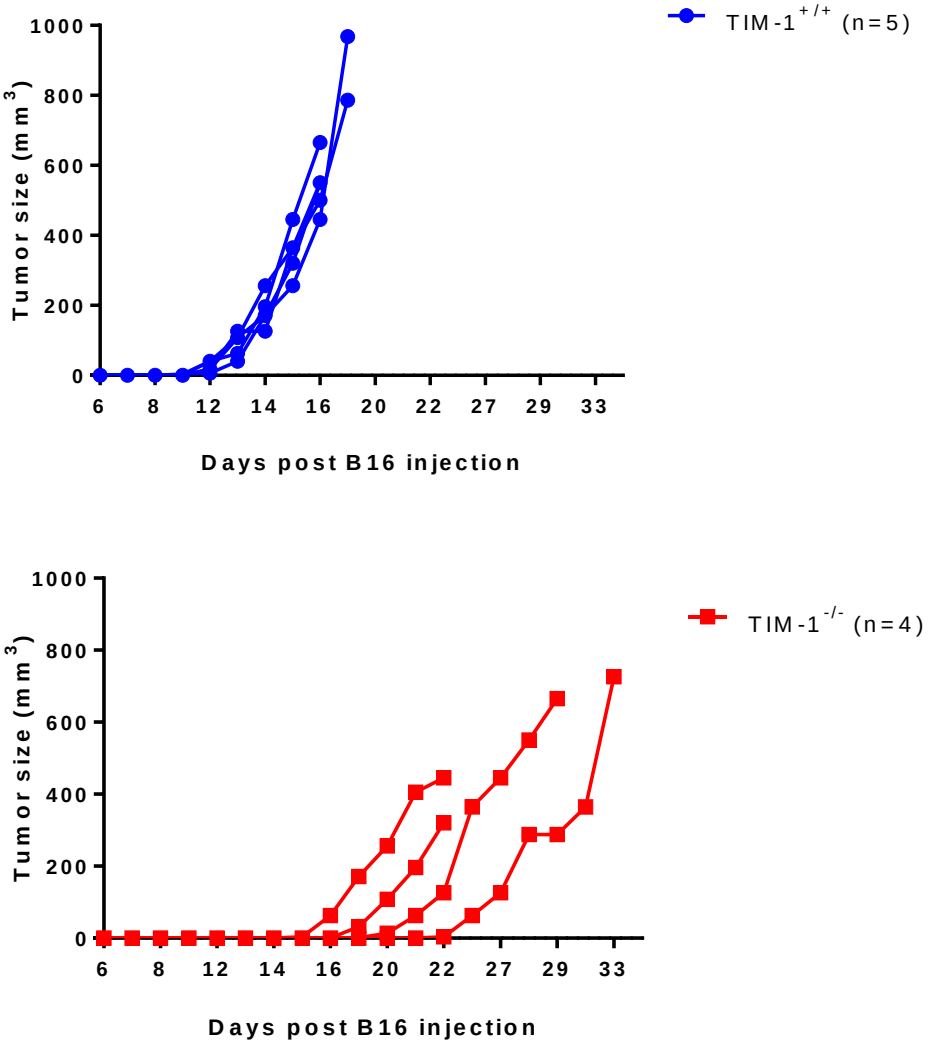
Submitted by: Kinchlea, Will D
on behalf of the Animal Care Committee
University Council on Animal Care

## Appendix B. Supplementary Figures

**A.**



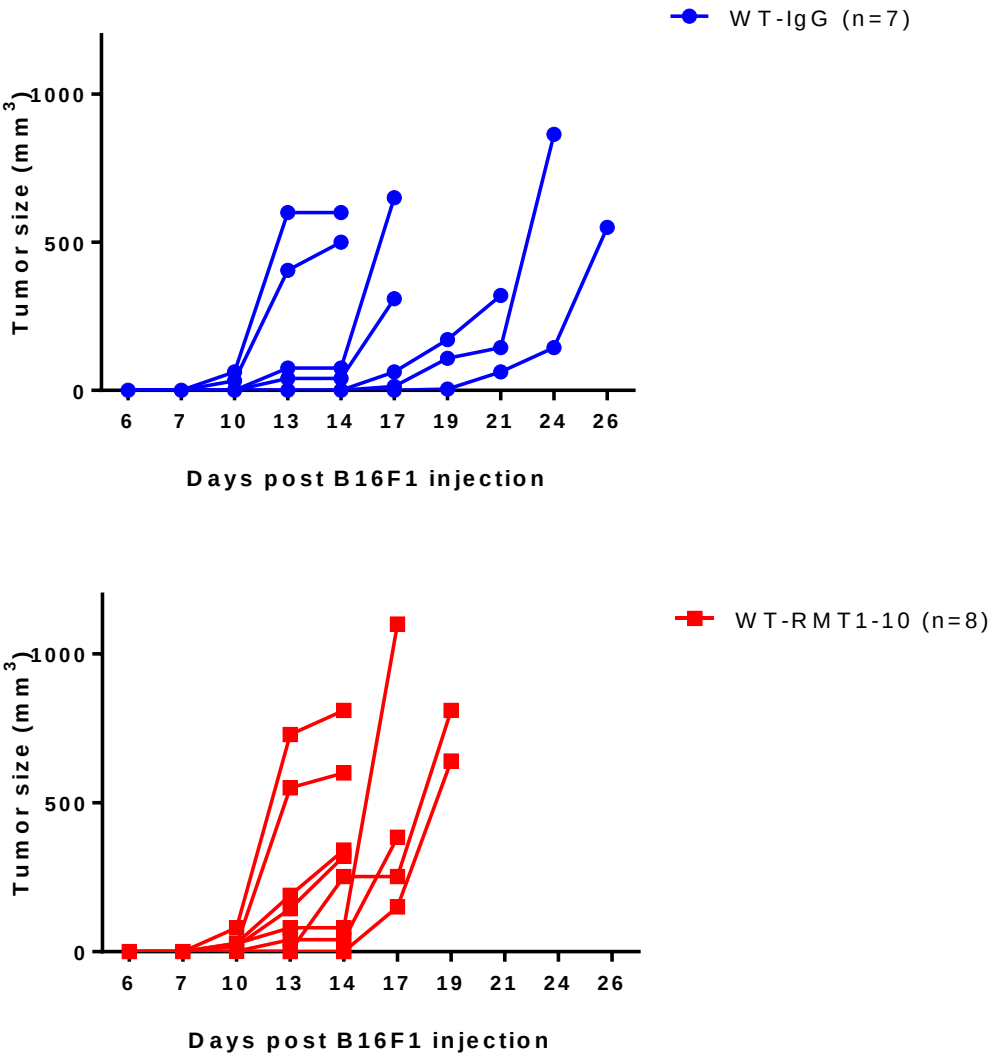
**B.**




**Supplementary Figure 1. Delayed B16F1 melanoma tumour growth and improved survival in immunocompetent Tim-1-deficient mice.**

Tim-1-expressing and Tim-1-deficient male C57BL/6 mice at 8-10 weeks old were subcutaneously inoculated with $5x10^5$ B16F1 melanoma. Endpoints were determined by incidence of cachexia, tumour ulceration, or a mean tumour diameter exceeding 10mm. **(A)** Spider plots depicting tumour growth from individual mice. **(B)** Spider plots depicting tumour growth from individual mice in the repeated experiment.

**A.**

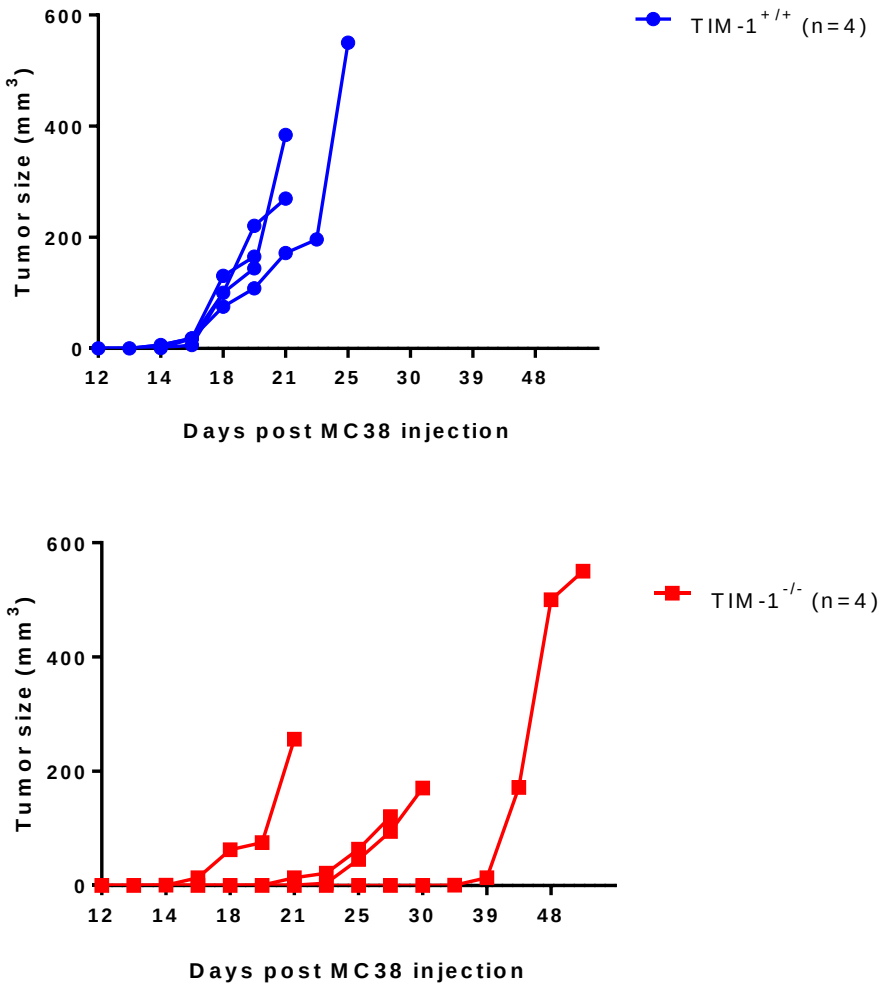



**Supplementary Figure 2. Effect of anti-Tim-1 monoclonal antibody, RMT1-10, on B16F1 melanoma tumour growth and survival in Tim-1-expressing mice.**

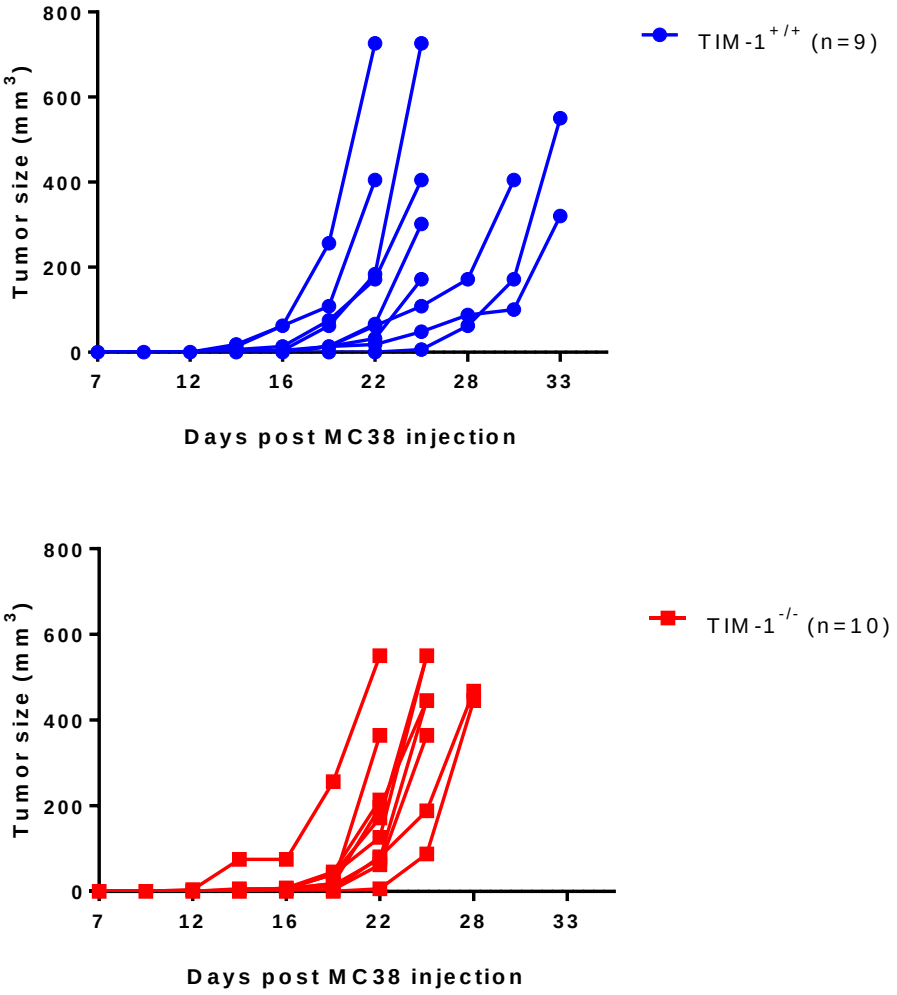
Tim-1-expressing mice received either 300ug of RMT1-10 (n=8) or 300ug of IgG control (n=7) intraperitoneally on days -1, 0, and every 3 days relative to subcutaneous B16F1 melanoma administration. Endpoints were determined by incidence of cachexia, tumour

ulceration, or a mean tumour diameter exceeding 10mm. **(A)** Spider plots depicting tumour growth from individual mice.

**A.**


600
400
200
0
Tumor size (mm3)
12
14
18
21
25
30
39
48
Days post MC38 injection
TIM-1+/+ (n=4)
TIM-1-/- (n=4)

**B.**




**Supplementary Figure 3. Effect of MC38 colorectal adenocarcinoma on Tim-1-expressing and Tim-1-deficient mice.**

Tim-1-expressing and Tim-1-deficient male C57BL/6 mice at 8-10 weeks old were subcutaneously inoculated with $1x10^5$ MC38 colorectal adenocarcinoma. Endpoints were determined by incidence of cachexia, tumour ulceration, or a mean tumour diameter exceeding 10mm. **(A)** Spider plots depicting tumour growth from individual mice. **(B)** Spider plots depicting tumour growth from individual mice in the repeated experiment.

# Curriculum Vitae

| | |
|---|---|
| **Name:** | Ingrid Hon |
| **Post-secondary Education and Degrees:** | The University of Western Ontario<br>London, Ontario, Canada<br>2014-2018 BSc. – Medical Sciences (Honors Specialization in Microbiology & Immunology with distinction) |
| | The University of Western Ontario<br>London, Ontario, Canada<br>2018-Present MSc. – Microbiology & Immunology |
| **Honours and Awards:** | Lawson Internal Research Fund Studentship<br>2019 |
| | The Western Scholarship of Excellence<br>2014 |
| **Related Work Experience:** | Graduate Teaching Assistant<br>The University of Western Ontario<br>2020 |
| **Conferences:** | Infection and Immunity Research Forum 2017 – London, Ontario |

**Abstract Title:** Elucidating The Role Of Host T Cell Immunoglobulin And Mucin Domain-1 (Tim-1) In Anti-Tumour Immunity – *Ingrid Hon and Lakshman Gunaratnam*